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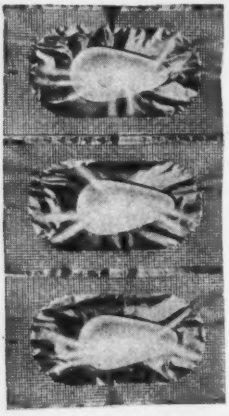

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ENDOCRINOLOGY

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THE DETERMINATION OF FORMALDEHYDOGENIC CORTICOIDS IN GUINEA PIG URINE

A. K. HALAMA

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THE ratio adrenal weight/body weight shows a wide variation in different mammals. It is largest in the guinea pig, viz. approximately 0.1% as compared with 0.01% in man (Hartmann and Brownell, 1949). Further advantages in favour of the guinea pig as a laboratory animal in adrenal research are that it is cheap and easy to keep and handle.

Recently Burstein (1952) presented a modification of the method for determination of formaldehydogenic corticoids in urine originally used by Doughaday, Jaffe and Williams (1948). This modification has been checked by Rosian (1954) and found suitable for a prolonged study of adrenal cortical activity in the guinea pig. It must be borne in mind, however, that this method of determination does not cover the total excretion of corticoids; only that fraction of adrenal cortical hormones, which liberate formaldehyde on periodic acid oxidation, is assayed quantitatively.

The present investigation was carried out in order to learn if changes of the adrenocortical activity in stress can be evidenced by estimating the urinary excretion of formaldehydogenic corticoids in guinea pigs. Exposure to cold was used as stressor.

METHODS

Male guinea pigs of a mixed breed, 8 to 18 months old and weighing 350 to 600 gm., received a basal diet of 80 gm. white bread and 200 gm. vegetables daily. As it is known that plant pigments impair the corticoid determination care was taken to exclude them from the diet. White cabbage, white turnips, china cabbage and cauliflower (stems without leaves) proved suitable. Urine collection and corticoid assay were carried out according to Burstein (1952) whose extraction procedure was considered superior to that of Kassenaar and Huis in 't Veld (1954), at least for work with small quantities of urine (as in the case of the guinea pig).

Received April 29, 1955.

The experimental animals were kept at room temperature; of these four were chosen at random for stress experiments and placed for half an hour daily in the refrigerator at +4° C. Later on, this exposure to low temperature was increased to one full hour per day (Fig. 1).

Animals that died during the investigation period were autopsied and examined macroscopically.

RESULTS

Normal values. In apparently healthy, untreated male guinea pigs a mean value of 42.8 ± 10.5 μg. desoxycorticosterone equivalent from one hundred 17 hours' urine samples was obtained. This figure agrees well with the value found by Burstein (1952), i.e. 45 μg.

Desoxycorticosterone was used to construct a calibration curve and in these estimations the error of the method was found negligible. The rela-

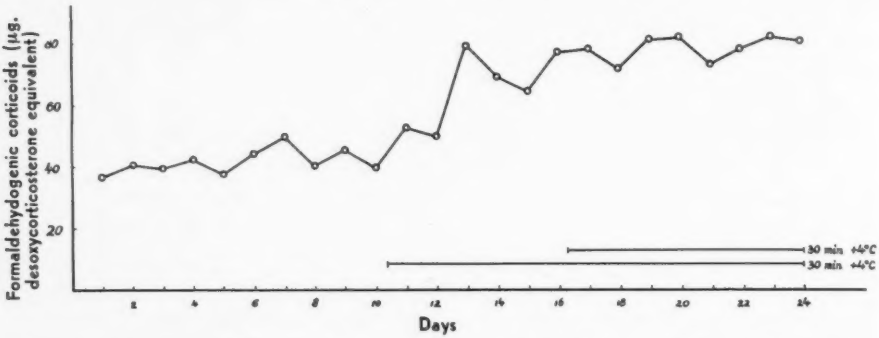

FIG. 1. Adrenocortical hormone content of 17 hours' urine samples in stress (mean of four male guinea pigs). Horizontal lines above abscissa indicate time of stressor action.

tive large standard error may, therefore, entirely be attributed to the variation between animals and 17 hours' urine specimens. In spite of constancy of diet with respect to quantity and composition, the collected urine volumes varied from 2 to 76 ml. in different animals during the investigation period of four months.

To anticipate a possible objection the following may be stated: chromotropic acid which was used as absorbent for the liberated formaldehyde, is known to be affected by carbon dioxide. To eliminate this source of error the distillation was carried out in nitrogen atmosphere. The chromotropic acid-sulfuric acid solution was always prepared freshly, immediately before use. Control estimations of known amounts of desoxycorticosterone were frequently run parallel with the samples and consistently gave satisfactory results.

Premortal corticoid elevation. In the course of this investigation 11 guinea pigs died. The post mortems revealed in six cases slight gastroenteritis, in two cases pneumonia and in one case purulent pleurisy. Two animals

were found extremely cachectic without any other visible changes. Seven of the animals showed a marked increase in urinary formaldehydogenic corticoid excretion during the last one or two days before death (Table 1).

Urinary corticoids during stress. In an initial control period the corticoid values of four male guinea pigs were observed and the animals afterwards exposed to cold as described above. After a latency period of two days the corticoid contents of the 17 hours' urine samples increased and remained constantly elevated thereafter.

Before the stress effects became manifest, the mean corticoid excretion during a 17 hours period was 48.5 μg. desoxycorticosterone equivalent

TABLE 1. RISE IN FORMALDEHYDOGENIC CORTICOID EXCRETION IN THE URINE OF GUINEA PIGS SHORTLY BEFORE DEATH (EXPRESSED IN μg. DESOXYCORTICOSTERONE EQUIVALENT)

Days before death							Day of death
7	6	5	4	3	2	1	0
						22	**74**
			42	56	51	92	**138**
					45	36	**87**
					21	52	**86**
				37	29	41	**60**
41	39	53	65	43	50	68	**110**
				37	32	41	**59**

and rose two days after the first exposure to stress to an average of 77.0 μg. This level was maintained rather constantly. Increase of the stressor time from 30 minutes to 1 hour only led to a minor further rise of adrenocortical hormone excretion (Fig. 1).

The mean formaldehydogenic corticoid daily excretions before and after exposure to stress differed by 28.5 μg. desoxycorticosterone equivalent (95% confidence limits 15.7 and 41.3). Student's t-test was applied, and the difference between the values before and after onset of stress changes was found significant at the 99.9% level of probability.

Influence of injury on corticoid excretion. One of the animals kept under normal conditions escaped during the feeding period and accidentally entered a neighbouring box containing a much larger male, known as a fighter. At discovery it had already suffered the following injuries: skin lesion about 30 mm. in diameter in left jugular region and bite wounds on face and back.

In spite of these lesions the animal did not seem to be very much affected. It took food soon after re-isolation and very attentively tried to lick its wounds. Blood loss was negligible, general activity unimpaired. Recovery took place a few days later.

The urine collected during the 17 hours' period following the accident had a formaldehydogenic corticoid content of 153 μg. desoxycorticosterone

equivalent. This figure only fell to 126 on the second day, and it took five days until normal values were reached again (Fig. 2).

DISCUSSION

The results were satisfactory with regard to the changes in the urinary excretion of formaldehydogenic corticoid hormones during stress, injury and the days preceding death. The minimal sensitivity of about 15 μg. desoxycorticosterone equivalent as reported by Rosian (1954) makes this

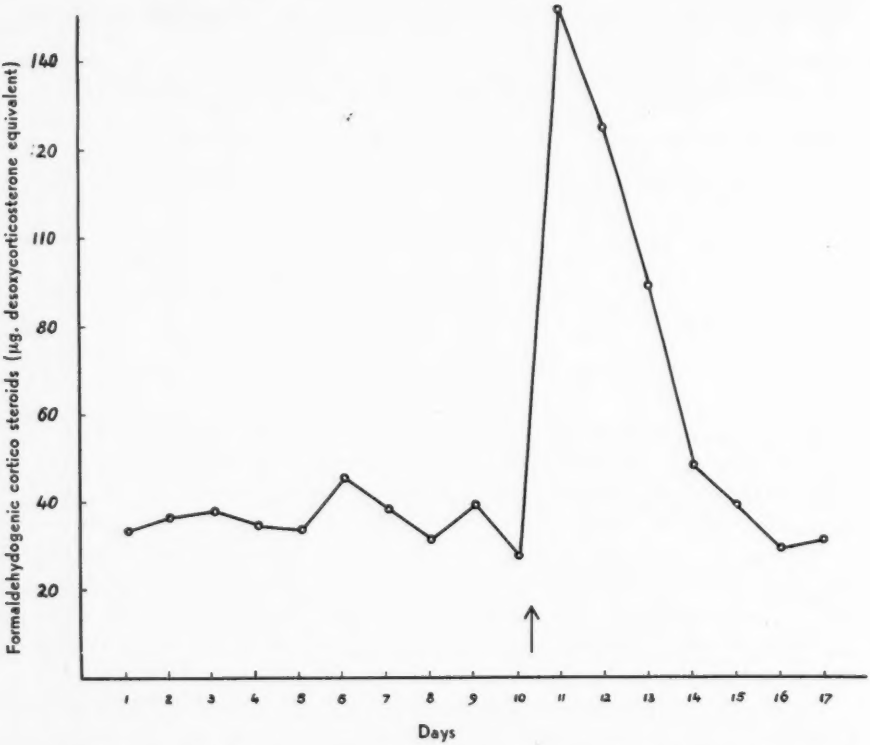

FIG. 2. Excretion of adrenocortical hormones in a male guinea pig after injury (urine collection period 17 hours daily). Arrow indicates time of injury.

modified formaldehyde determining method a valuable tool in the study of adrenocortical activity in a cheap and common laboratory animal, viz. the guinea pig.

An apparent disadvantage is the relatively large standard error, resulting in a coefficient of variation of 23%, despite uniform housing and feeding of the test animals during the entire investigation period. Distillation under nitrogen, in order to avoid impairing effects on the colour generating

reaction between formaldehyde and chromotropic acid by carbon dioxide, did not lead to an appreciable reduction of this value.

Eleven deaths occured in the first part of the investigations. Later on, when the vegetable supplement was more varied—although kept constant in weight—the test animals remained in good health for weeks.

A great advantage of the corticoid assay after Burstein (1952) is its simplicity as compared with the 17-ketosteroid determination chosen by Oertel and Rein (1954) for testing adrenocortical activity in scorbutic guinea pigs. In the present investigations all laboratory work was done by the author alone without any auxiliary personnel. The time necessary for working a series of 6 to 8 urine samples was about 5 hours including the intervals required for oxidation and colour development. Few other chemical corticoid determinations can be performed so quickly. It may be mentioned that the financial expenses are rather small, the main item being the costs for ether and chloroform (parts of the extraction mixture).

SUMMARY

Determinations of formaldehydogenic corticoids were carried out in urine samples of male guinea pigs under normal and stress conditions. A marked rise after prolonged exposure to cold, after injury and shortly before death was observed. The method used in this investigation proved simple, cheap and sensitive.

Acknowledgment

I am indebted to Dr. F. X. Wohlzogen, Institute of Physiology, University of Vienna, for criticism.

REFERENCES

Burstein, S.: *Endocrinology* **50:** 412. 1952.

Doughaday, W. H., H. Jaffe and R. H. Williams: *J. Clin. Endocrinol.* **8:** 166. 1948.

Fisher, R. A. and F. Yates: Statistical Tables for Agricultural, Biological and Medical Research, 3rd ed., Oliver & Boyd, Edinburgh 1948.

Hartmann, F. A. and K. A. Brownell: The Adrenal Gland, Lea & Febiger, Philadelphia 1949.

Kassenaar, A. A. H. and L. G. Huis in 't Veld: *Nederl. Tijdschr. Geneesk.* **98:** 623. 1954.

Oertel, G. and H. Rein: *Arzneimittel-Forsch.* **4:** 513. 1954.

Rosian, F.: Vet. Inaug.-Diss., Vienna 1954.

THE RENAL β-GLUCURONIDASE RESPONSE TO ANDROGENS[1,2]

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THE ability of testosterone to enhance kidney β-glucuronidase activity was observed in white Swiss and C3H mice (Fishman, 1951) and was later investigated in detail by Fishman and Farmelant (1953) in several strains of inbred mice. Testosterone propionate administration (7 mg. divided over 14 days) resulted in elevated kidney β-glucuronidase levels which were from four to thirteen-fold greater than control levels, depending on the strain employed in the study. Liver β-glucuronidase activity remained unchanged. Coincident with the rise in kidney β-glucuronidase activity, a marked excretion in the urine of β-glucuronidase was noted by Riotton and Fishman (1953).

It is evidently desirable to arrive at some method of quantitating the activity of androgenic steroids on the basis of a biochemical analysis rather than by morphological or weight changes in a given target tissue. With regard to the renal β-glucuronidase response the following questions required consideration.

Within what range do kidney β-glucuronidase levels fluctuate in normal mice, and does this variation relate to the strain of the animal? In this regard, white Swiss mice of mixed strain were found to exhibit an undesirable degree of variability in kidney β-glucuronidase values in contrast to mice of several inbred strains.

What is the basis of selecting the strain of animal for assay purposes? Previous work (Fishman and Farmelant, 1953) had indicated that of seven mouse strains studied, A/Cloudman mice yielded the highest value for testosterone-propionate-stimulated renal β-glucuronidase. This is a "high" β-glucuronidase strain (Morrow, Greenspan and Carroll, 1949, 1950, 1951; Fishman and Farmelant, 1953) whose resting renal β-glucuronidase

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[1] This investigation was supported by grants-in-aid from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, Bethesda, Maryland (C-915), and by an institutional grant from the American Cancer Society, Inc., New York. Both the support and the gifts of many rare steroid compounds by the Upjohn Company are gratefully acknowledged. We are indebted for supplies of steroids also to the Worcester Foundation for Experimental Biology, The Smith, Kline and French Company and the Organon Company.

[2] This paper was presented at the 36th meeting of the Endocrine Society, June 17, 1954 in San Francisco.

fluctuated between narrow limits (±10%). For these reasons A/Cloudman mice were chosen.[3]

The question of the sex of the experimental animals and the state of their gonads next arose. For certain strains of mice (AKR, BAF_1, CAF_1, DBA, Fishman and Farmelant, 1953; Morrow *et al.*, 1951) kidneys of male animals are richer than those of the female sex. In other strains (C3H, 129) there is no sex difference. Male mice of the A/Jax strain possess more β-glucuronidase activity than female mice (3140±300 units/gm. for kidneys of 10 male mice versus 1920±140 units/gm. for 10 female mice). It was decided to utilize male mice of the A/Jax strain. Inasmuch as the increase in renal β-glucuronidase induced by testosterone propionate was seen in both intact and castrated mice (Fishman and Farmelant, 1953), the intact animal was chosen.

Does the diet influence the β-glucuronidase response? If it is reasoned that the androgen-induced rise in renal β-glucuronidase activity is a reflection of an increase in enzyme protein (Sie and Fishman, 1953), the nutrition of the test animals would appear to be a variable in the proposed test system. Accordingly, experiments were done to assess the influence of the proportions of protein, fat, carbohydrate, B-vitamins and of food intake on the kidney β-glucuronidase response to testosterone propionate. Results of these experiments are reported in this paper.

On the basis of the above considerations, therefore, experimental conditions now have been defined for grading the renal β-glucuronidase response to various C-19 and C-21 steroids. These conditions and the results obtained in over seventy-five compounds are the subject matter of this communication.

METHODS AND EXPERIMENTAL CONDITIONS

Animals. Male mice of the A/Jax strain weighing 18 to 22 gm. were secured from the Jackson Memorial Laboratories at Bar Harbor, Maine.

Mode of administration. The use of pressed tablets of steroids was avoided because of the known variation in the rate of steroid absorption from subcutaneously implanted pellets. Except in a few instances, the steroids could be dissolved completely in peanut oil (10 mg. per 1 ml.) at room temperature. In the case of the more insoluble compounds, the mixture was heated to 100° for several minutes. If solution was incomplete, the well-suspended mixture was injected. The dry crystalline compounds before solution were in each case triturated in a 25 cc. Erlenmeyer flask with a solid rounded glass rod (diameter 4 mm.) until the product was finely divided. This is a necessary step in the technique. The area of loose skin below the neck was chosen as the site of subcutaneous injection.

Diet. The synthetic diet chosen for maintaining mice for steroid testing was the "25% protein, 25% fat" regime, which was constituted in parts per 100 as follows: casein (GBI acid precipitated), 25; Mazola oil, 15; Crisco, 5; cod liver oil, 5; sucrose, 22; dextrin,

[3] The A/Cloudman strain as bred and supplied by the Jackson Memorial Laboratories, Bar Harbor, Maine bears the name, A/Jax.

22; "Alphacell" (Nutritional Biochemicals Corp.), 2; salt mixture (GBI, No. 2), 4; vitamin B mixture, 4 ml. per 100 gm. of diet. 500 ml. of stock B-vitamin mixture was composed as follows: pyridoxine HCl 0.05 gm.; thiamine HCl 0.05 gm.; riboflavin/0.05 gm.; nicotinic acid 1.5 gm.; calcium pantothenate 0.2 gm.; *i*-inositol 0.2 gm.; *p*-aminobenzoic acid 0.2 gm.

In Table 1 and Figure 1 are presented data concerning the β-glucuronidase response to testosterone propionate by animals maintained on this and other diets. The composition of the synthetic diets other than the one chosen for the testing of steroids was arrived at by increasing the carbohydrate component as required when either the fat or protein was reduced, the source and amount of the other ingredients remaining unchanged. The stock diet was composed of a nutritionally adequate mixture of animal and plant ingredients (Purina Fox checkers).

Synthetic diets have the advantage of lacking estrogens, which often contaminate mixed stock diets. Under the conditions of the experiment, the renotrophic effect of testosterone was noted but was not sufficiently uniform to warrant its further study in relation to the testing of steroids for renal β-glucuronidase stimulating power.

TABLE 1. EFFECT OF DIET ON KIDNEY β-GLUCURONIDASE RESPONSE TO TESTOSTERONE PROPIONATE

A/Jax male mice in groups of 10 were placed on these various diets and test groups received 7 mg. of testosterone propionate in peanut oil over a 14 day period, controls being given peanut oil only. On the fifteenth day, the animals were sacrificed and the analyses performed on the tissues as described in the text.

Experimental Diet	Treatment	β-Glucuronidase activity (units/gm.)		
		Kidneys	Liver	Spleen
Lab Chow	Control	3,730 ±860	3,060 ±240	4,820 ±430
	T. P.	42,210 ±1,700	3,880 ±244	5,430 ±450
5% Protein 25% Fat	Control	2,970 ±546	6,280 ±1,030	6,460 ±1,240
	T. P.	42,170 ±4,000	4,180 ±1,730	6,160 ±1,220
25% protein 5% fat	Control	2,530 ±177	4,040 ±870	4,980 ±910
	T. P.	38,700 ±1,630	5,810 ±1,500	5,760 ±520
25% protein 25% fat	Control	4,610 ±461	3,530 ±950	4,470 ±410
	T. P.	51,710 ±5,060	3,000 ±1,040	5,870 ±1,400
25% protein 25% fat restricted intake	Control	2,150 ±182	2,330 ±430	5,290 ±730
	T. P.	46,130 ±6,510	2,480 ±300	6,450 ±1,200
18% protein 15% fat	T. P. without B-vitamins	35,300 ±5,350	4,140 ±625	7,300 ±790
	T. P. With B-vitamins	40,780 ±4,080	6,230 ±450	5,730 ±1,320

Conditions for steroid testing. Groups of 10 male mice were placed on the 25% protein, 25% fat diet for 17 days, injections being initiated on the fourth day. Each mouse received subcutaneously 0.1 cc. of peanut oil containing 1.0 mg. of steroid by injection every other day for 14 days. Urine was usually collected daily and pooled and assayed for β-glucuronidase activity in the course of the study (Riotton and Fishman, 1953). On the fifteenth day the animals were sacrificed and kidney β-glucuronidase was determined on specimens of kidneys of each animal according to Fishman and Farmelant (1953), using the substrate, phenolphthalein glucuronic acid (Fishman, Springer and Brunetti, 1948; Talalay, Fishman and Huggins, 1946).

Renal β-Glucuronidase response to steroids. In Table 2 are illustrated in detail typical data obtained with steroids selected at random from the present series, which exhibited varying degrees of activity. The urinary β-glucuronidase increased to very high levels in the more active compounds, and it is possible to predict from the urinary excretion whether or not one is dealing with an active steroid.

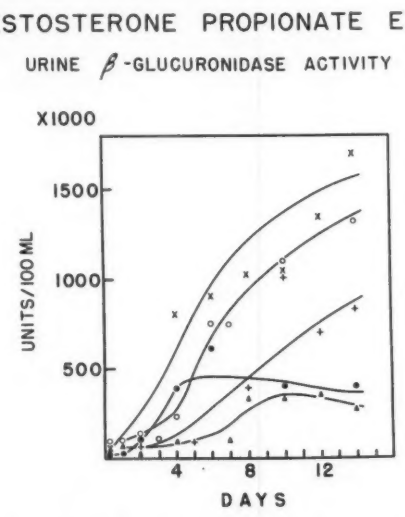


FIG. 1. Urinary β-glucuronidase changes in animals maintained on various diets receiving 7 mg. of testosterone propionate divided over 14 days. x—x, 25% protein, 25% fat diet; o—o, 25% protein, 5% fat; +—+, restricted intake of 25% protein, 25% fat; .—., 5% protein, 25% fat; ▲—▲, lab chow.

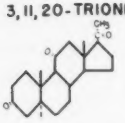
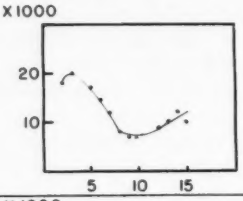
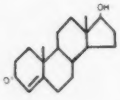
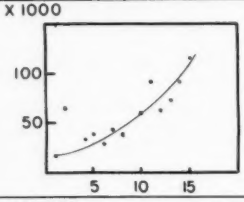
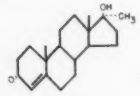
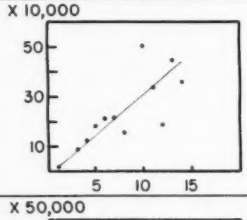
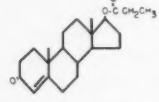
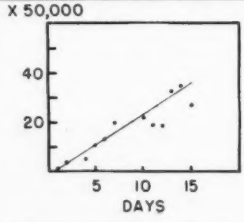
Table 3 lists the results obtained with a large variety of steroids. For convenience in comparing these results the average kidney β-glucuronidase activity of the control group (3140 units/gm.) receiving peanut oil alone has been assigned a value of 1.0, referred to as the kidney β-glucuronidase factor. The factor assigned to a given steroid was computed by dividing the mean kidney β-glucuronidase activity of the test group by 3140.

In Table 4 are given the statistical data concerning differences in the response of selected compounds to which significance is attached in the interpretation of the results.

RELATION OF RESPONSE TO CHEMICAL STRUCTURE OF STEROIDS

Inasmuch as androgens appeared to be the most potent group of steroids so far studied, the significant findings (Table 4) are pointed out in rela-

TABLE 2. TYPICAL DATA OBTAINED WITH REPRESENTATIVE STEROIDS, STANDARD CONDITIONS BEING EMPLOYED

STEROID	KIDNEY β-GLUCURONIDASE ACTIVITY U/G.		URINARY β-GLUCURONIDASE UNITS/100 ML
ALLOPREGNANE 3,11,20-TRIONE 	4400	2990	
	4100	2540	
	2400	2870	
	3150	2100	
	3200		
	MEAN 3080	S.D. ±710	
TESTOSTERONE 	8900	6880	
	9280	11,050	
	7350	9440	
	9300	9550	
	7180	11,600	
	MEAN 9050	S.D. ±1490	
METHYLTESTOSTERONE 	26,950	20,100	
	14,500	13,900	
	19,750	21,750	
	18,000	27,000	
	16,500	20,200	
	MEAN 19,850	S.D. ± 4320	
TESTOSTERONE PROPIONATE 	47,500	52,700	
	48,500	53,800	
	48,900	46,900	
	49,500	52,300	
	54,600	50,400	
	MEAN 50,510	S.D. ± 2560	

tion mainly to the structure of testosterone (Δ^4-androstene-17β-ol,-3-one), which induced a three-fold kidney β-glucuronidase response (factor 2.9).

Configuration and grouping at position 17. The 17-OH epimer of testosterone, and its 17-keto-derivatives and the 17-vinyl-, and ethinyl-substituted compounds showed reduced ability to increase kidney β-glucuronidase. On the other hand, the 17-methyl derivative exhibited greater activity than either testosterone or its 16-methyl derivative.

Introduction of oxygen at position other than 3 or 17. Except for 11β-hydroxytestosterone, oxygenation at positions 6, 11 or 14 produced less active compounds.

TABLE 3. RESULTS OF GRADING STEROIDS WITH RESPECT TO THE RENAL β-GLUCURONIDASE RESPONSE

	Kidney Glucuronidase Factor
Miscellaneous Steroids	
None	1.0
Cholesterol	1.0
Cedilanid	1.0
Hydroxybisnorcholenic acid	0.8
Estradiol	0.8
Stilbestrol	0.7
Progesterone	0.8
Testosterone and Its Derivatives	
Testosterone	2.9
Epitestosterone	1.2
Vinyltestosterone	1.9
17-methyltestosterone	6.3
16-methyltestosterone	2.8
Testosterone enamine	1.7
Androstanedione-3,17	1.5
Adrenosterone	1.5
Δ^4-androstene-3,17-dione	1.2
11β-hydroxy-4-androstene-3,17-dione	1.0
Etiocholane-3,11,17-trione	1.0
Androstane 17β-ol-3-one	5.4
Androstane 3β-ol-17-one	1.3
$\Delta^{9,11}$-dehydrotestosterone	2.8
6β-hydroxytestosterone	1.4
$\Delta^{3,5}$-cycloandrostane-6-methoxy-17β-ol	1.1
19-nortestosterone	3.9, 4.2
11α-hydroxytestosterone	1.9
11β-hydroxytestosterone	2.7
11-ketotestosterone	1.0
14α-hydroxytestosterone	0.9
11β-hydroxytestosterone-17-propionate	2.6
11-ketotestosterone propionate	4.3
Testosterone diethylacetate	5.9
Testosterone-β-bromopropionate	11.4
Testosterone-β-cyclopentylpropionate	12.4
19-nortestosterone cyclopentylpropionate	15.7
Testosterone propionate	16.0, 16.3, 16.7
5-Androstene Series	
Δ^5-androstene-3β,-17β-diol	2.0
Δ^5-androstene-3β,17β-diol diacetate	1.8
Δ^5-androstene-3β,17β-diol-3-acetate-17-benzoate	1.2
Δ^5-androstene-3β,17β-diol-17-benzoate	1.1
Δ^5-androstene-3β-ol-17-one	1.3
17β-carbethoxyamino-5-androstene-3β-ol	1.0
3β-acetoxy-17β-hexahydrobenzoxy-5-androstene	1.0
Δ^5-androsten-3β,17β-diol-3-(2-tetrahydropyranyl) ether-17-cyclopentylpropionate	1.1
$\Delta^{3,5}$-androstadiene-17-one,3 ethoxy	1.8
$\Delta^{3,5}$-androstadienedione	2.2
17α-ethinyl-5-androstene-3β,17β-diol	1.4
17α-methyl-5-androstene-3β,17β-diol	9.5
17α-methylandrostane-3α,17β-diol	14.2
Pregnane Derivatives	
11α,17α,21-trihydroxy-Δ^4-pregnene-3,20-dione	1.5
11β,17α-dihydroxy-Δ^4-pregnene-3,20-dione	2.1
Cortisone acetate	1.9
17α-hydroxy-11-ketoprogesterone	2.0
Substance S-acetate	1.8
17α-hydroxyprogesterone	1.9
Δ^5-pregnene-3β,17α diol-20-one-3 acetate	1.7

TABLE 3—(*continued*)

	Kidney Glucuronidase factor
3β-11α-dihydroxyallopregnane-20-one	1.3
11β-hydroxyprogesterone	1.0
11α-hydroxyallopregnane-3,20-dione	1.2
3α,17α-dihydroxy-21-bromopregnane-11,20-dione	0.9
3α,17α-dihydroxypregnane-11,20-dione	1.3
17α-hydroxy-21-acetoxypregnane-3,11,20-trione	0.9
11α-hydroxypregnane-3,20-dione	1.0
3α-hydroxypregnane-11,20-dione	1.1
3β-hydroxyallopregnane-11,20-dione	0.9
3α,20β-dihydroxypregnane-11-one	0.8
3α,17α-dihydroxy-21-acetoxypregnane-11,20-dione	0.9
3α,11β-dihydroxypregnane-20-one	1.3
11β,17α-dihydroxypregnane-3,20-dione	1.4
Desoxycorticosterone acetate	1.2
Pregnenolone	1.0
6β,11α-dihydroxyprogesterone	0.9
11α-acetoxyallopregnane-3,20-dione	0.9
11α-acetoxypregnane-3,20-dione	0.9
3β-hydroxy-11α-acetoxy-allopregnane-20-one	0.8
3α,11α-dihydroxypregnane-20-one	1.1
17αOH, 21-acetoxy-4 bromopregnane-3,11,20-trione	1.3
11-ketoprogesterone-3,20-ethylene diketal	1.0
Pregnane-3α,11α,20 triol	1.3
Pregnane-3,20 dione	0.9
Progesterone enamine	0.7
Epicorticosterone	0.7
14α-hydroxydesoxycorticosterone	0.7
14α,17α-dihydroxy-21-acetoxy-4-pregnene-3,20-dione	0.8
Cortisone cyclopentylpropionate	1.0
3α,11β,17α-trihydroxypregnane-20-one	0.9
4-chloro,17α-hydroxy-21-acetoxypregnane	0.7
3β,17α,21-trihydroxyallopregnane-11,20-dione-21-acetate	0.8
11β-hydroxypregnane-3,20-dione	0.8
4-chloro-11β,17α-dihydroxypregnane-3,20-dione-3-ethylene glycol ketal	0.8
3β-ol-20-one-16,17-epoxide-Δ^5-pregnene	0.7
17-hydroxypregnenolone	1.0
Δ^4-pregnenetriolone diacetate	1.0
Allopregnane-3,20 dione-21-ol	1.3
11α-hydroxyprogesterone	0.9
11-ketoprogesterone	0.9
Allopregnane-3,11,20-trione	1.0
Pregnane-3,11,20-trione	0.9

Position 3 and α,β-unsaturation in ring A. The absence of the double bond of testosterone enhanced its activity, cf. androstane-17β-ol,3-one and androstene-17β-ol,3-one. Both the 3-keto and Δ^4 double bond groupings are present in many inactive compounds of this series and are therefore not regarded as important features of the molecule with regard to the renal β-glucuronidase response.

Absence of the 19-methyl group. Nortestosterone was a more active compound than testosterone under the present experimental conditions.

Esterification of the 17β-OH group. Invariably, esterification of testosterone enhanced its ability to stimulate renal β-glucuronidase. This was not always true for the esterified modified testosterones. Thus, while the propionate of 11β-OH testosterone was no more active than the unesterified steroid, 11-ketotestosterone propionate was potent, while 11-ketotestosterone exhibited no activity.

TABLE 4. STATISTICAL SIGNIFICANCE OF DIFFERENCES IN KIDNEY GLUCURONIDASE FACTORS OF PAIRS OF STEROIDS SELECTED FOR COMPARISON[4]

Experiment No.	Steroid	Kidney glucuronidase factor	Comparisons of Exp no. vs Exp no.		*T.*	P.
1	Control	1.0				
2	Testosterone	2.9	1	2	8.65	<0.01
3	Epitestosterone	1.2	2	3	5.03	<0.01
4	Androstanedione-3,17	1.5	2	4	2.78	0.013
5	Adrenosterone	1.5	2	5	3.58	<0.01
6	Δ^4-Androstene-3,17-dione	1.2	2	6	3.81	<0.01
7	11β-hydroxy-4-androstene-3,17-dione	1.0	2	7	6.40	<0.01
8	Etiocholane-3,11,17-trione	1.0	2	8	5.65	<0.01
9	Androstane-3β-ol,17-one	1.3	2	9	4.90	<0.01
10	Vinyl-testosterone	1.9	2	10	2.63	0.017
11	Ethinyl-testosterone	1.3	2	11	4.55	<0.01
12	Methyltestosterone	6.3	2	12	3.90	<0.01
13	16-methyltestosterone	2.8	12	13	3.63	<0.01
14	6β-hydroxytestosterone	1.4	2	14	4.34	<0.01
15	11α-hydroxytestosterone	1.9	2	15	3.66	<0.01
16	11-ketotestosterone	1.0	2	16	6.8	<0.01
17	Androstane-17β-ol,3-one	5.2	2	17	2.5	0.02
18	Nortestosterone*	4.1	2	18	3.0	<0.01
19	Δ^5-Androstene-3β, 17β-diol	2.0	1	19	4.43	<0.01
20	Δ^5-Androstene-3β, 17β-diol, 3-acetate, 17-benzoate	1.2	19	20	3.05	<0.01
21	Δ^5-Androstene-3, βol, 17-one	1.3	19	21	2.31	0.035
22	17β-carbethoxyamino-5-androstene-3β-ol	1.0	19	22	3.8	<0.01
23	3β-acetoxy-17β-hexahydrobenzoxy-5-androstene	1.0	19	23	3.0	<0.01

* The data of two groups of mice each are pooled here.

Δ^5-Androstenediol, some of its derivatives and esters. Δ^5-Androstenediol was active and lost activity when the 17β-OH was esterified with benzoic acid (but not acetic acid), or when it was oxidized to the ketone group. Also, altering the 17β-OH group as in 17β-carbethoxyamino-Δ^5-androstene-3β-ol or in 3β-acetoxy-17β-hexahydrobenzoxy-Δ^5-androstene abolished activity. The 17α-methyl derivative (methylandrostenediol) was highly active, an effect corresponding qualitatively to the introduction of the 17α-methyl group into testosterone. The 17α-ethinyl derivative of Δ^5-androstene, 3β, 17β-diol was inactive.

Pregnane derivatives. Seven of the 49 compounds tested exhibited renal β-glucuronidase factors of 1.5 to 2.0. These apparently active C_{21}-pregnane substances possess in common the following functional groups: 3-keto, Δ^4, 17αOH, 20-keto. The remainder of the pregnane steroids were essentially negative.

DISCUSSION

The mouse renal β-glucuronidase response to androgen is a reproducible phenomenon. Fishman (1951, 1955) and Fishman and Farmelant (1953)

[4] This table concerns only differences in the kidney β-glucuronidase factors between selected pairs of steroids where statistical treatment of the data was considered necessary.

have consistently found that the administration of testosterone, its propionate or other androgens (MAD) invariably increased mouse renal β-glucuronidase. Accompanying this elevation in kidney β-glucuronidase was a marked urinary excretion of the enzyme (Riotton and Fishman, 1953). The extension of this study to a large number of steroids has provided information regarding the specificity of this phenomenon and some of the factors which influence it.

Nature of the renal β-glucuronidase response. This response is considered reasonably specific for androgens, inasmuch as estrogens, progesterone and a large number of pregnane derivatives give negative results. The small effects seen in a few corticoid compounds are considered to arise secondarily from androgenic products of the metabolism of these substances (Harrison, Leman, Munson and Laidlaw, 1955; Sokal, *et al.*, 1954) or by other more indirect pathways.

The influence of the composition of the diet on the ability of the kidney β-glucuronidase to respond to administered testosterone propionate is interesting. Although the nutrition experiments were designed solely to help select an optimal diet for this study, nevertheless it appears that the maximum level of renal β-glucuronidase following testosterone propionate required an unrestricted intake of a diet rich in protein and fat, supplemented with B vitamins. It would appear the dietary conditions may influence the synthesis of new β-glucuronidase protein, if this is the case.

The renal β-glucuronidase response is regulated to a large extent by the genetic background of the animal. Thus, in a study of 7 inbred mouse strains, the administration of testosterone propionate under standard conditions produced renal responses varying from four to thirteen-fold (Fishman and Farmelant, 1953). Recently, Fishman (1955) found that the offspring of a variety of crosses between high and low β-glucuronidase strains, for example, A/Cloudman and C3H, showed greater or lesser responsiveness depending on the presence or absence of the dominant tissue glucuronidase gene, G (Law, Morrow and Greenspan, 1952).

Grading of steroids on the basis of the renal β-glucuronidase response. The purpose of the present study has been to survey a variety of steroids in order to learn which ones possessed the property of enhancing kidney β-glucuronidase in A/Jax mice under standard conditions. Control data obtained in experiments with and without inactive steroids were uniform, and within each group the standard deviation in per cent ranged from ±10 to 20. Active compounds are defined as substances which raise the kidney β-glucuronidase factor to a value of 2 or more, the percentage standard deviation usually being of the same order as in the controls, although occasionally rising to ±25%.

Three orders of activity were discernible; thus, steroids with factors of 2 to 4, 5 to 10, and 10 to 16. As the activities increased, only a change in the factor of 2 or 3 indicates a real difference in the potencies of two

compounds; for example, the potency of testosterone-β-bromopropionate (factor 11.4) is not different statistically from testosterone β-cyclopentylpropionate (factor 12.4), both, however, being weaker than testosterone propionate (factor 16.0). Moreover, it is suspected that in this high range of activity, the observed responses may be duplicated with smaller amounts of steroid; that is, the response noted may be occurring at the insensitive plateau of the dose-response curve. This is a subject presently being investigated with the aim of defining still further conditions which may make the renal β-glucuronidase response to androgens a basis for accurate biochemassay.[5]

The question of the significance of the renal β-glucuronidase response. Whether the mouse renal β-glucuronidase response is a property correlating with either, both or neither of the virilizing or protein anabolic functions of androgens is an important question. At the present time, this question cannot be answered accurately. Part of the difficulty arises from the inherent overlap in virilizing and anabolic functions of androgens and from the many interpretations possible from the published figures of others relating to androgenic potency. Factors of species, vehicle, biologic end-point, period of administration, etc. are variables which make comparisons only educated guesses.

Some clues may be forthcoming from considerations of the relationship of functional groups of the steroids and their biological and biochemical activity. Position 17 of androgens appears to be an area of marked importance with regard to the renal β-glucuronidase response. Although this is true to some extent in the case of the virilizing property of androgens (for example, testosterone versus epitestosterone), there are several striking instances of a separation of biological activities. Thus, the introduction of the 17α-methyl group into Δ^5 androstene-3β,17β-diol yields MAD, which is a weak androgen (Gordon *et al.*, 1951; Homburger *et al.*, 1950), or the absence of the C-19 angular methyl group of testosterone (Hershberger *et al.*, 1953) as in 19-nortestosterone reduces its secondary sex organ stimulating power. Yet both of these compounds rated higher than testosterone in the renal β-glucuronidase response, and both have been reported to possess a favorable ratio of protein anabolic (based either on renotrophic action or levator ani muscle weight) to virilizing potencies (weight of prostate and/or seminal vesicles) (see Gordon *et al.*, 1951; Homburger *et al.*, 1950; and Hershberger *et al.*, 1953). It should be noted that Barnes, Stafford, Guild and Olson (1954) consider that MAD has no preferential myotrophic activity.

A consideration of the results of other workers yields additional ex-

[5] The term "biochemassay" is intended to signify an assay procedure based on the measurement of a chemical principle in a tissue stimulated by a given biological agent in contradistinction to bioassay, which usually depends upon changes in weight or morphological appearance of the tissue.

amples of the paramount importance of position 17 of androgens and the existence of a lack of strict correlation of androgenicity with effects on other tissues. Thus, Gaunt *et al.* (1953) reported that methylandrostenediol was more effective than testosterone in preventing cortisone inhibition of the adrenal. From their investigation of a large number of steroids, it was concluded that the presence of the 17β-OH group in the molecule was the function correlating best with the adrenal-atrophy preventive effect. Huggins and Jensen (1954), working with the hypophysectomized rat, described position 17 of C-19 steroids as critical for inducing growth of the uterus, vagina and prostate. It is curious also that nortestosterone was more uterotrophic than testosterone and that 11α-OH 6β-OH, and 17 ketotestosterones were less active than testosterone (cf. renal β-glucuronidase response).

Influence of androgens on mouse renal enzymes. Effects of androgens on renal enzymes have been noted before, but the phenomena were niether as marked or as specific as the renal β-glucuronidase response appears to be. Thus, mouse kidney arginase is increased by the administration of androgen (Kochakian, 1947), cortisone (Kochakian and Robertson, 1950) and estradiol-17β (Kochakian, Garber and Bartlett, 1948), whereas administration of testosterone propionate to castrated male mice restored to normal the renal d-amino acid oxidase previously reduced by castration (Clark, Kochakian and Fox, 1943). Increases in acid phosphatase of the kidney of mice given testosterone propionate were noted by both Kochakian (1947) and Rabinovitch (1952), whereas the average cell "alkaline" phosphatase is diminished (Rabinovitch, 1952).

SUMMARY

Experimental conditions have been established which make it possible to obtain uniform and reproducible figures for mouse kidney β-glucuronidase activity in A/Jax strain mice which include a synthetic diet rich in protein and fat supplemented with B-vitamins. A survey of a large number of steroids has been completed in order to identify compounds active in eliciting the renal β-glucuronidase response. Androgens as a group were usually positive, yielding from two to sixteen-fold increases in renal β-glucuronidase, 7 out of 49 pregnane and corticoid derivatives were weakly active, whereas estrogens and progesterone were without effect. Considering testosterone as the reference steroid (three-fold response), the following changes in the molecule usually enhanced potency: esterification of the 17β-OH, introduction of the 17α-methyl group, reduction of the Δ^4 double bond and elimination of the 19 angular methyl group. The following alterations reduced potency; replacement of the 17β by a 17α-OH group, oxidation to 17 ketones and introduction of an 11α, 14α, or a 6β OH group. In the Δ^5 androstenediol series, great enhancement of activity was seen in the 17α-methyl compound (MAD), whereas blocking the 17β-OH

group or its oxidation to 17-keto derivatives reduced potency. Attention has therefore been focused on position 17 of the steroid molecule as a particularly important area in the molecule which has a determining effect on the steroid's potency with regard to the renal β-glucuronidase response. These results are discussed in relation to other renal enzymes and non-sex organ effects of androgens.

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REFERENCES

BARNES, L. W., R. O. STAFFORD, M. E. GUILD AND K. J. OLSON: *Proc. Soc. Exp. Biol and Med.* **87:** 35. 1954.

CLARK, L. C., JR., C. D. KOCHAKIAN AND R. P. FOX: *Science* **98:** 89. 1943.

FISHMAN, W. H.: In Advances in Enzymology, XVI (Interscience Publishers, Inc., New York) p. 390. 1955.

FISHMAN, W. H., B. SPRINGER AND R. BRUNETTI: *J. Biol. Chem.* **173:** 449. 1948.

FISHMAN, W. H.: *Ann. N. Y. Acad. Sci.* **54:** 548. 1951.

FISHMAN, W. H. AND M. H. FARMELANT: *Endocrinology* **52:** 536. 1953.

GAUNT, R., C. H. TUTHILL, N. ANTONCHAK AND E. OPPENHEIMER; *Arch. Exper. Path. u. Pharmakol.* **219:** 91. 1953.

GAUNT, R., C. H. TUTHILL, N. ANTONCHAK AND J. H. LEATHEM: *Endocrinology* **52:** 407. 1953.

GORDON, G. S., E. EISENBERG, H. D. MOON AND W. SAKAMOTO: *J. Clin. Endocrinology* **11:** 209. 1951.

HARRISON, J. H., C. LEMAN, P. L. MUNSON AND J. C. LAIDLAW: *New Eng. J. of Med.* **252:** 425. 1955.

HERSHBERGER, L. G., E. G. SHIPLEY AND R. K. MEYER: *Proc. Soc. Exp. Biol. and Med.* **83:** 175. 1953.

HOMBURGER, F., I. FORBES AND R. DESJARDINS: *Endocrinology* **47:** 19. 1950.

HUGGINS, C. AND E. V. JENSON: *J. Exp. Med.* **100:** 225–241. 1954.

KOCHAKIAN, C. D.: In Recent Progress in Hormone Research (Academic Press, Inc., New York) **1:** 177. 1947.

KOCHAKIAN, C. D. AND E. ROBERTSON: *Arch. Biochem.* **29:** 114. 1950.

KOCHAKIAN, C. D., E. E. GARBER AND M. N. BARTLETT: *Am. J. Physiol.* **155:** 265. 1948.

LAW, L. W., A. G. MORROW AND E. M. GREENSPAN: *J. Natl. Cancer Inst.* **12:** 909. 1952.

MORROW, A. G., E. M. GREENSPAN, AND D. M. CARROLL: *J. Natl. Cancer Inst.* **10:** 657. 1949; **10:** 1199, 1950; **11:** 663. 1951.

RABINOVITCH, M.: *Separata de Folia Clinica et Biologica* **18**(2): 115. 1952.

RIOTTON, G. AND W. H. FISHMAN: *Endocrinology* **52:** 692. 1953.

SIE, H. G. AND W. H. FISHMAN: *Cancer Research* **13:** 590. 1953.

SOKAL, J. D., P. K. BONDY, P. J. COSTA, C. L. DEMING AND M. D. HARVARD: *Yale J. of Biol. and Med.* **26:** 345. 1954.

TALALAY, P., W. H. FISHMAN AND C. HUGGINS: *J. Biol. Chem.* **166:** 757. 1946.

OXYTOCIC ACTIVITY OF DIENCEPHALON AND TUBER CINEREUM OF NORMAL AND HYPOPHYSECTOMIZED RATS

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THE presence of oxytocic, antidiuretic and vasopressor activities has been reported for the hypothalamus (Abel, 1924; Trendelenburg, 1932; Hild, 1951 a, b; Hild and Zetler, 1953). These activities have been identified with corresponding activities of the posterior lobe of the hypophysis (Perez, 1954; Zamorano, 1954).

According to Bargmann and Scharrer (1951) the hormones from the neurohypophysis are produced by the supraoptic nucleus and carried to the posterior lobe of the hypophysis, which is a sort of discharge area. This hypothesis agrees with Hild and Zetler's (1953) observations, who stated that after section of the supraoptic-hypophyseal tracts the Gomori granules tend to accumulate at the central end of the tract.

If the hormones are carried from the supraoptic nucleus to the posterior lobe of the hypophysis, the distribution of the active principles should change whenever the connections are severed. In order to study this problem we have tried to determine the oxytocic potency of the supraoptic nucleus and of the tuber cinereum region in hypophysectomized rats.

METHODS

Ten experimental groups were set up for the determination of the oxytocic activity. At least 4 control and 4 hypophysectomized animals were included in each group.

Hypophysectomy was performed through the floor of the cranial cavity. Immediately after the operation the animals were injected with 10 ml. of a solution of 4.5% NaCl. The diuresis was measured daily.

The animals were killed 5 to 14 days after hypophysectomy and extracts from equivalent amounts of anterior hypothalamus and tuber region were prepared immediately. The material was ground with micronized quartz sand, adding 0.5 ml. per 100 gm. rat body weight of water acidified to pH 3. The sand was removed by centrifugation at 2000 r.p.m. for 15 minutes. The weight of the endocrine organs was recorded.

The titration of the oxytocic activity was carried out on uteri of rats in diestrus, after neutralization of the extract. A Tyrode solution prepared according to Gaddum *et al.* (1949) and containing 2 mg. of atropine per liter was used in the bath. The volume of extract added varied between 0.1 and 0.8 ml. and comparison of the activities was attained by dilution.

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RESULTS

The hypophysectomized rats showed increased water ingestion and diuresis. At necropsy some remnant of hypophyseal tissue, deprived of oxytocic activity, was occasionally located around the perforation of the skull. A decrease in weight of the thyroid, the adrenals, the seminal vesicles and the uterus was observed.

No difference in the content of oxytocic substances was found in the anterior hypothalamus, which showed an average activity corresponding to 0.5 mU of oxytocin. A constantly greater oxytocic activity was present in the tuber region of hypophysectomized rats as compared to normal

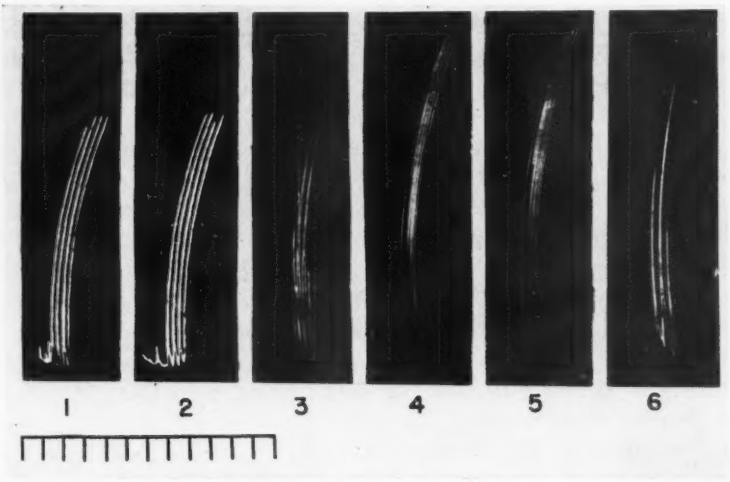

FIG. 1. Contractions of rat uterus in Tyrode solution. Additions: 1. 0.3 ml. extract of anterior hypothalamus from hypophysectomized rat. 2. 1 ml. of anterior hypothalamus from normal rat.

3 and 6. 0.2 ml. extract of tuber from normal rat.

4 and 5. 0.2 ml. extract of tuber from hypophysectomized rat.

Time scale: 5 minutes intervals.

rats (Fig. 1). In the tuber of normal rats the oxytocic activity was equivalent to 0.25 mU, while in the hypophysectomized rats it was over 2 mU, frequently 4 mU.

DISCUSSION

Our findings could be interpreted according to the hypothesis of Bargmann and Scharrer (1951). They do not reveal either the causes for the increased activity or the nature of the oxytocic agent. It is possible that the increase in activity might be due to accumulation of substances coming from the hypothalamus or to a hyperplasia of the infundibulum or the median eminence.

SUMMARY

The oxytocic activity of the tuber of hypophysectomized rats was found to be higher than that of control animals.

REFERENCES

ABEL, J. J.: *Bull. Johns Hopkins Hosp.* **25**: 203. 1924.
TRENDELENBURG, P.: *Arch. exp. Path. u. Pharmakol.* **131**: 42. 1932.
HILD, W.: *Virchow's Arch.* **319**: 526. 1951.
HILD, W.: *Acta Neuroveg.* **3**: 81. 1951.
HILD, W. AND G. ZETLER: *Zeitschr. f. das ges. exp. Med.* **120**: 236. 1953.
PEREZ, A.: Tesis de Grado, Universidad de Chile. 1954.
ZAMORANO, B.: Tesis de Grado, Universidad de Chile. 1954.
BARGMANN, W. AND E. SCHARRER: *American Scientist*, **38**: 255. 1951.
HILD, W. AND G. ZETLER: *Pflügers Archiv.* **257**: 169. 1953.
GADDUM, J. H., W. S. PEART AND M. VOGT: *J. of Physiol.* **108**: 467. 1949.

THE DEVELOPMENT OF THE HYPOTHALAMO-NEUROHYPOPHYSIAL SECRETORY SYSTEM IN THE CHICK EMBRYO

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THE study of neurosecretion in vertebrates and invertebrates was greatly stimulated by Bargmann's application of the chrom-hematoxylinphloxine stain of Gomori to the study of neurosecretory substance in the hypothalamo-neurohypophysial tract of the dog and cat. (Bargmann, 1949; Gomori, 1941). Since then this technique has been used with gratifying results in studies of neurosecretion in many animal species. One of the many problems investigated has been that of the ontogeny of the neurosecretory tracts of the hypothalamus.

Wingstrand (1954) has reported on the early formation of neurosecretory substance staining with Gomori's stain in the chick embryo. He attempted to correlate the histological findings with bioassays for antidiuretic substance in the embryonic hypothalamus. Although there was physiological evidence of antidiuretic substance in the hypothalamus by the tenth day of incubation he found no histological evidence of neurosecretion until the 13th or 14th day. This led Wingstrand to propose either that the stainable material was not associated with the antidiuretic hormone which is physiologically evident early in the development of the hypothalamo-neurohypophysial tract, or that it was present in too slight concentration to be detected by the histological techniques at hand.

The present study of the ontogeny of neurosecretion was undertaken to serve as a standard of reference for other experiments on neurosecretion in the chick embryo. The results differ considerably from those reported by Wingstrand and certain additional information has been gained concerning the perhaps allied matter of ependymal secretion.

MATERIAL AND METHODS

Forty-nine white leghorn chick embryos were incubated at 99¾ degrees F. Beginning at 48 hours incubation embryos were sacrificed daily by stripping the membranes away and immersing in fixative. Embryos of 2 to 7 days incubation time were simply immersed *in toto*; those from 8 to 20 days were decapitated at the time of immersion; the heads of those from 15 to 20 days were sliced open on one side at the time of killing to permit greater penetration of fixative.

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Three fixatives were used: (1) Bouin's solution, (2) formol-saline (Bodian, 1937), and (3) formol-saline-sublimate (Pearse, 1952). With a few exceptions one chick was fixed in each solution for each day of incubation. After one day of fixation the head of each chick was sliced in a sagittal plane to leave a mid-section measuring about 2 to 3 mm. in thickness. These portions were left in the fixative one more day and were then placed in 80 per cent ethyl alcohol and left for a period of 39 to 61 days. They were then dehydrated in alcohol, cleared in xylol, and embedded in paraffin. Serial sections were cut at 6 microns with every 10th section mounted. A few of the larger specimens were sectioned at 7 microns.

Duplicate sets of serial sections were prepared from each specimen by mounting adjacent sections from the paraffin ribbon on the different slides. These sets were stained according to the following plan:

Bouin's solution

1. Chrom-hematoxylin-phloxine (Gomori, 1941)
2. Periodic acid-Schiff (Wilson and Ezrin, 1954)

Formol-saline

1. Chrom-hematoxylin-phloxine
2. Silver nerve fiber stain (Rogers, Pappenheimer and Goettsch, 1931)

Formol-saline-sublimate

1. Chrom-hematoxylin-phloxine
2. Masson trichrome stain

A few triplicate sets of slides were prepared for Schiff staining without prior oxidation with periodic acid to serve as controls for the periodic acid-Schiff (PAS) stain.

RESULTS

Effect of the three fixatives used on the chrom-hematoxylin-phloxine stain

Differentiation of the neurosecretory substance was greatest with Bouin's solution. This fixative also decalcified the skulls of the larger embryos. Formol-saline proved very inadequate for studying neurosecretory substance with the chrom-hematoxylin-phloxine stain because of several factors, the chief of these being excessive phloxinophilia of the tissue, poor differentiation of the neurosecretory substance, and poor preservation of cytologic detail.

Neurosecretory substance

48 hours. The future supraoptic region shows no evidence of fiber tract development or differentiation of neuroblasts. The region of the future infundibulum and sacculus is richly cellular and there is abundant mitotic activity. There is no demonstrable neurosecretory substance in this region of the diencephalon.

68 hours. There is very slight widening of the future chiasmatic and supraoptic regions with proliferation and enlargement of neuroblasts. The future sacculus shows no evidence of invasion of nerve fiber tracts and still lies in contact with Rathke's pouch (Fig. 1). The neurones of the

supraoptic region have a slight bluish cast in the cytoplasm near the nuclei but no granules are visible with the light microscope.

97 hours. The supraoptic region is now expanding rapidly with an increase in neurones and their processes. There is an extension of fibers toward the sacculus; however, the latter is still largely devoid of fibers. There is now a thin zone of mesenchymal tissue and capillaries separating the infundibulum and Rathke's pouch (Fig. 3). The cells of the supraoptic region at this stage show large accumulations of dark blue neurosecretory substance which sometimes fill the cytoplasm and extend well into the axone hillocks. Some cells have a small cap of neurosecretory material lying on one side of the nucleus (Fig. 4). The same material stains with PAS reagents.

5 days. The supraoptic region has now undergone enormous growth. There is a nerve fiber layer extending to the tip of the sacculus (Fig. 5). There is an increase in the neurosecretory substance which is also "PAS positive" in the supraoptic region. This material can be seen in axones at this stage (Fig. 6). In addition, the ependymal cells in the distal portion of the sacculus are beginning to form clumps of phloxinophilic material in their cytoplasm about the nuclei and between the nuclei and the ventricular borders of the cells. This material stains lightly with the PAS stain.

6 days. The sacculus now shows a fine granular deposit of neurosecretory material in the fiber layer near the base (Fig. 7). This stains lightly with the PAS stain.

7 days. There is continued passage of neurosecretory material in increased amount through axones from the supraoptic region (Figs. 8, 9). PAS granules are beginning to appear in a few cells of the anterior lobe. The material in the ependymal cells is still accumulating and stains dark blue at this stage. The appearance is indistinguishable from that of the neurosecretory substance of the hypothalamic-neurohypophysial tract.

8 days. The Masson trichrome stain shows the early differentiation of basophils in the pars distalis. There are no acidophils.

10 days. There is marked increase in the neurosecretory material deposited in the base of the sacculus.

12 days. There is an increase in the intensity of the PAS stain and the neurosecretory substance in the sacculus ependymal cells (Fig. 10).

14 days. There are now excrescences forming on the ventricular faces of the ependymal cells in the distal portion of the sacculus. These vary from round projections to droplets lying free near the cells. Some have adhered to one another and formed a string of droplets resembling a bacterial chain. Nissal bodies are now forming in the neurones of the hypothalamus.

17 days. The ependymal excrescences are still forming (Fig. 11). By

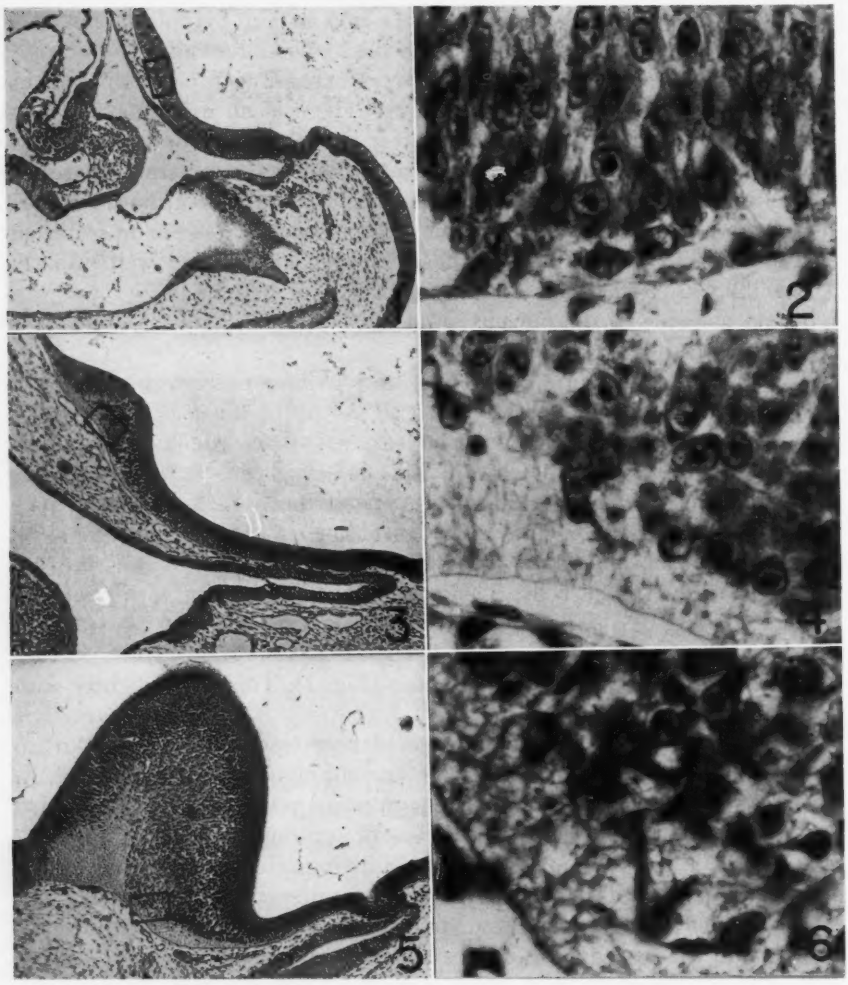

FIG. 1. Base of the brain of the 68 hour chick embryo showing the region of the future optic chiasm and supraoptic nucleus. There is early evagination of the floor of the 3rd ventricle forming the infundibulum. Rathke's pouch lies adjacent to the floor of the 3rd ventricle. The rectangular area is enlarged in Figure 2. Chrom-hematoxylin-phloxine. × 73

FIG. 2. High magnification of the rectangular are marked on Figure 1 showing early spreading apart of cells in the future supraoptic region. Chrom-hematoxylin-phloxine. × 933

FIG. 3. Base of the brain of the 97 hour chick embryo showing the result of proliferation of neurones in the supraoptic region. There is also beginning separation of Rathke's pouch from the infundibulum by the ingrowth of mesenchyme. The rectangular area is enlarged in Figure 4. Chrom-hematoxylin-phloxine. × 73

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this time intensely "PAS positive" cells are seen in the anterior lobe. There is no acidophilic differentiation.

18 days. The sacculus is multilobed. By now small hyaline hematoxylinophilic bodies are present which resemble classical Herring bodies. The ependymal excrescences have ceased forming and the ependymal cells are now almost depleted of neurosecretory material; that which remains is a residual finely granular dispersion between the nuclei and the ventricular borders of the cells. Many of the nuclei are reduced in size.

20 days. There is essentially no change. There is no differentiation of acidophils by the pars distalis.

Argentophilia of the nerve fibers.

As early as the 97 hour stage there is faint staining of nerve fibers in the hypothalamus with the technique used. However, there is little or no increase in the intensity of the stain in this region and in the infundibular tracts throughout the remaining embryonic life. The fibers elsewhere, especially those of the cerebellum and brain stem, develop intense argentophilia early in embryonic life (Figs. 12a, 12b).

DISCUSSION

The results show that neurosecretory substance is formed in the chick hypothalamus by 68 hours of incubation coinciding with the earliest differentiation of neuroblasts in the supraoptic region. By 97 hours of incubation neurosecretory substance is seen in the axones as they begin to extend toward the sacculus. By the 6th day nerve fibers have extended to the tip of the sacculus and neurosecretory material is present there. It seems therefore that the early formation of neurosecretory substance keeps pace with the maturation of neurones, and the passage of neurosecretory substance through the axones closely follows the extension of the axones through the infundibulum and sacculus.

Wingstrand reported that there was no histological evidence of neurosecretion earlier than the 13th day of incubation. The present results are not in agreement with his finding. The reasons for this discrepancy are

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FIG. 4. High magnification of the rectangular area in Fig. 3 showing the presence of neurosecretory substance in the cytoplasm of neurones. The neurosecretory substance extends into the axone hillocks in many cells. Chrom-hematoxylin-phloxine. × 933

FIG. 5. Base of the brain of the 5 day chick embryo showing the enormous growth by this time, of the chiasmatic and supraoptic regions. There is evidence of extension of nerve fibers along the base of the infundibulum. The proliferation of a capillary bed over the median eminence is causing further separation of Rathke's pouch and the infundibulum. The rectangular area is enlarged in Figure 6. Chrom-hematoxylin-phloxine. × 73

FIG. 6. High magnification of the rectangular area in Figure 5 showing greater concentration of neurosecretory substance in the supraoptic neurones as well as the presence of the substance in the axones. Chrom-hematoxylin-phloxine. × 933

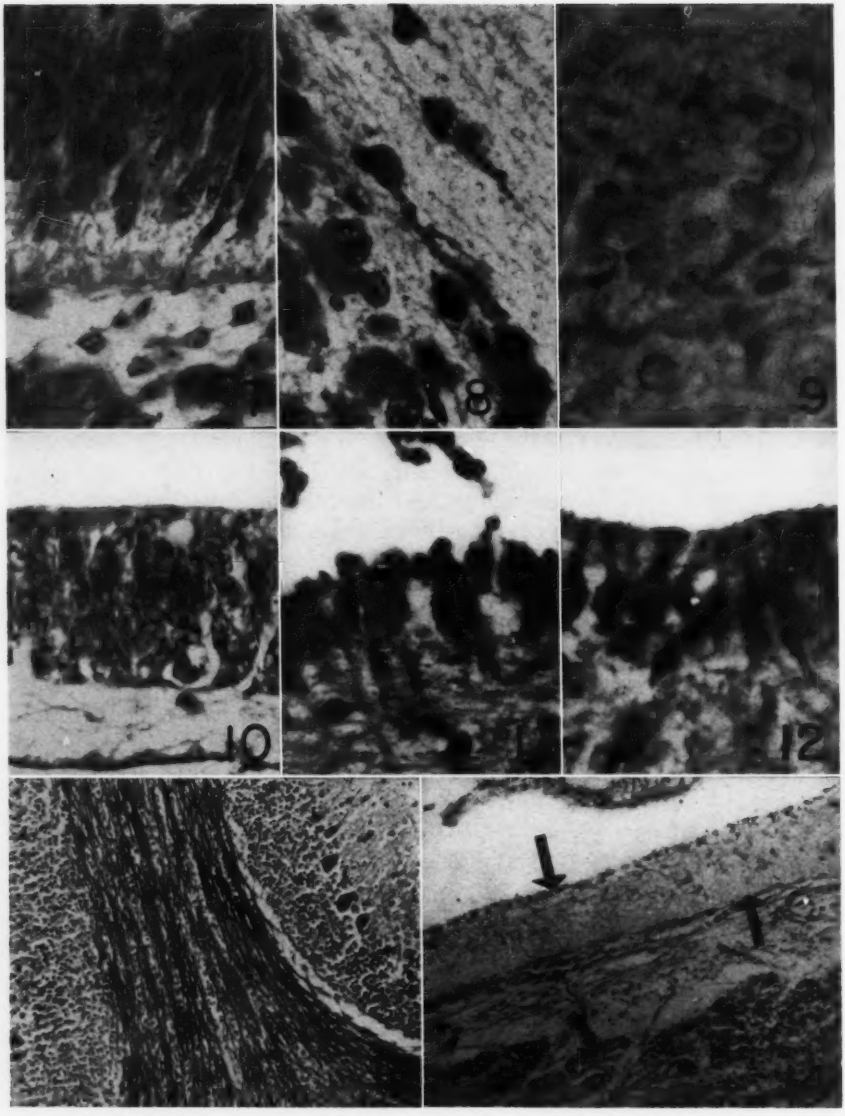

FIG. 7. Base of the sacculus in the 6 day chick embryo showing an increased density near the base of the fiber layer caused by finely granular neurosecretory substance. Chrom-hematoxylin-phloxine. ×895

FIG. 8. Supraoptic neurones in the 7 day chick embryo showing neurosecretory substance in passage through axones. Note the nodular appearance of the axone in the center of the field. Chrom-hematoxylin-phloxine. ×950

FIG. 9. PAS stain of the supraoptic region of the same chick represented in Figure 8

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not readily apparent. If one compares the histological results in this study with Wingstrand's bioassay data, the earliest histological evidence precedes bioassay evidence by about 5 days, or about 25 per cent of the entire span of embryonic life. It needs to be emphasized at this point that the substance stained by the chrom-hematoxylin-phloxine stain is not identical with antidiuretic hormone (Scharrer and Scharrer, 1954). However, it is probably intimately associated with antidiuretic hormone, possibly as a cement substance or carrier. The quantity of neurosecretory material present in the hypothalamo-neurohypophysial tract is generally believed to bear a direct relationship to the quantity of antidiuretic substance present (Scharrer and Scharrer, 1954).

The appearance of PAS-stainable material in general was found to coincide in time and location with the Gomori stainable material in the chick series. Sections treated with the Schiff reagent without prior oxidation with periodic acid failed to show a reaction in these areas. This would indicate that the Gomori-stainable neurosecretory substance is identical with or is associated with a glycoprotein.

The occurrence of ependymal excrescences localized in the sacculus has not previously been described to our knowledge. The formation of neurosecretory substance in the ependymal cells appears to be independent of the neurosecretory activity of the hypothalamo-neurohypophysial tract. The substance is predominantly phloxinophilic early, but it soon adopts hematoxylinophilia and resembles the neurosecretory substance of the hypothalamus. PAS-staining is again associated with the material both as to location and quantity. There is continual increase in the amount of the substance in the ependymal cells from about the 5th day of incubation until the 14th day when it begins to pass out of the cells into the cavity of the sacculus and infundibulum. This process stops between the 17th and 18th day of incubation when the cells are nearly depleted of neurosecretory

showing the faint selective staining with PAS of the material in neurones and axones. PAS. ×1067

FIG. 10. Sacculus of the 12 day chick embryo showing the dense accumulation of neurosecretory substance in the ependyma between the nuclei and the ventricular borders of the cells. The base of the sacculus fiber layer contains neurosecretory material probably derived from the hypothalamus. Chrom-hematoxylin-phloxine. ×950

FIG. 11. Sacculus of the 17 day chick embryo showing the forming and free ependymal excrescences. Note that some are stuck together in short strings. Chrom-hematoxylin-phloxine. ×950

FIG. 12. Sacculus of the 18 day chick embryo after the ependyma has ceased forming excrescences. The neurosecretory material has diminished in the ependymal cytoplasm. Chrom-hematoxylin-phloxine. ×950

FIG. 13. Cerebellum of a 19 day chick embryo showing intense argentophilia of the nerve fibers. Silver stain. ×120

FIG. 14. Region of the median eminence (indicated by arrows) in the same brain section used for Figure 13 showing the relatively faint argentophilia of nerve fibers in the hypothalamo-hypophysial tract. Silver stain. ×120

substance and have only a residual blue haziness to the cytoplasm. The significance of this process as well as the fate of the secreted material remains to be determined. Whether this substance is related to the known antidiuretic function of the hypothalamus can only be a matter of conjecture. This possibility might be initially investigated by performing assays for antidiuretic activity on the cerebrospinal fluid of the chick embryo during this stage of its development.

The formation of neurosecretory substance in the ependymal cells of the subcommissural organ has been described for every class of vertebrates (Wingstrand, 1954; Legait, 1946; Mazzi, 1952). This group of specialized ependymal cells underlying the posterior commissure extrudes fine strands of neurosecretory material which proceed caudad and coalesce to form Reissner's fiber (Wingstrand, 1954). This activity seems to bear no relation to that seen in the sacculus. It is not inconceivable that ependymal secretion occurs in still other locations and plays one or more specific hormonal roles.

The difference in silver staining by the hypothalamo-neurohypophysial tract fibers could be more satisfactorily discussed if we understood the chemical steps involved in this type of nerve fiber stain reaction. One might suppose that the presence of neurosecretory substance in the axone inhibits the reaction with silver. Stutinsky (1950) states that some hypophysial fibers do not stain with the Gomori stain but do stain with silver and proposes that this implies a qualitative difference between the neurone which contains neurosecretory substance and that which stains with silver. Beyond this there is no basis for further consideration until more is known of the chemical nature of the stain reaction as well as the chemistry of the neurosecretory substance.

SUMMARY

The brains of chick embryos in daily stages of incubation from 48 hours through 20 days were serially sectioned and stained with Gomori's chrom-hematoxylin-phloxine to demonstrate neurosecretory substance. Adjacent sections were stained with periodic acid-Schiff, a silver nerve fiber stain and a trichrome stain. Representative chicks were fixed with Bouin's solution, formol-saline, and formol-saline-sublimate. Bouin's solution was the best fixative for the Gomori stain.

Neurosecretory substance appeared in the neurones of the supraoptic region as early as 97 hours incubation and was present in the axones by 5 days incubation. The substance was found in the infundibulum by the 6th day, and it increased in concentration in the infundibulum throughout embryonic life.

Material staining with PAS coincided in time and location with the appearance of neurosecretory substance. This may indicate that the neurosecretory substance has a polysaccharide component.

The infundibular ependyma formed a cytoplasmic material staining with Gomori's chrom-hematoxylin-phloxine early in development and then excreted droplets of this material into the ventricular cavity from the 14th through the 17th day of incubation. The significance of this phenomenon is unknown.

Argentophilia is relatively slight in the embryonic chick hypothalamo-hypophysial tract as compared with certain other fiber tracts of the brain.

REFERENCES

BARGMANN, W.: *Klin. Wchnschr.* **27**: 617. 1949.

BODIAN, D.: *Anat. Rec.* **69**: 153. 1937.

GOMORI, G.: *Am. J. Path.* **17**: 395. 1941.

LEGAIT, E.: *C. rend. Soc. biol.* **140**: 543. 1946.

MAZZI: *Arch. Zool. Ital.* **37**: 445. 1952. (Concerns Gomori staining substance in the subcommissural organ of Cyclostomes, Selachians, Teleosts, and Urodeles.) Reference given in Wingstrand, 1954.

PEARSE, A. G. E.: *J. Path. & Bact.* **64**: 791. 1952.

ROGERS, W. M., A. M. PAPPENHEIMER AND MARIANNE GOETTSCH: *J. Exp. Med.* **54**: 167. 1931.

SCHARRER, E. AND B. SCHARRER: *Recent Progress in Hormone Research,* Academic Press, Inc., N. Y., v. 10. 1954. p. 183. (An extensive review with numerous references.)

STUTINSKY, F.: *C. rend. Soc. biol.* **144**: 1357. 1950.

WILSON, W. D. AND C. EZRIN: *Am. J. Path.* **30**: 891. 1954.

WINGSTRAND, K. G.: *Arkiv för Zoologi.* **6**: 41. 1954.

THE REPORTED INHIBITION OF DCA-SALT HYPERTENSION BY ANTIHISTAMINIC DRUGS: EFFECTS OF SANDOSTENE, PHENERGAN AND CHLORPROMAZINE[1]

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TREATMENT of uninephrectomized rats with desoxycorticosterone acetate (DCA) and salt results in renal, splanchnic, myocardial and cerebral vascular lesions identical with those of experimental renal hypertension and similar in many respects to the lesions of hypertensive disease in human beings (Selye, 1942; Masson *et al.*, 1949). The mechanism of formation of these lesions is unknown, except that arterial hypertension seems to be a contributory factor. That altered vascular permeability, with trapping in the vessel walls of a protein component of plasma might play a role in hyalinization and fibrinoid change in the arterioles is suggested in part by the microscopic appearance of some of these lesions. This possibility receives further support from the finding that desoxycorticosterone, in the presence of excess Na ion, increases permeability of vessels in preparations of isolated limbs (Tripod and Meier, 1952). The importance of increased permeability in this connection seems to have been confirmed by the observations that the administration of Phenergan [(1-dimethyl-amino-2′methyl, 1′ethyl-N-) dibenzo-p-thiazinehydrochloride] or Neo-antergan, (N-p-methoxy benzyl-N-dimethylaminoethyl-α-aminopyridine), drugs which decrease vascular permeability by their strong antihistaminic actions, inhibits both the lesions and the hypertension induced by DCA and salt in rats (Zacco and Ciasco, 1950; Guillemin and Fortier, 1951). A structurally unrelated antihistaminic, Sandostene (1-methyl-4-amino-N′-phenyl-N′-(2′-thenyl)-piperidin tartrate) has also been found to inhibit the hypertension and edema produced in rats given 2% saline to drink (Rothlin and Cerletti, 1953).

We have re-examined the effects of Phenergan and Sandostene and have also studied those of chlorpromazine (Thorazine) [10-(3-dimethyl-aminopropyl)-2-chlorphenothiazine] which, although of only slight anti-

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histaminic potency, is structurally related to Phenergan and is of considerable current interest. The effects of these compounds were tested under similar conditions in 2 series of experiments in 2 strains of rats. Since the results were negative in both cases, we will report here only those of the second series.

METHODS

Fifty-six male albino Sprague-Dawley rats weighing 100 to 135 gm. were uninephrectomized, divided into 8 groups with the same average body weight and treated as indicated in Table 1. There were 6 animals per group in the first 4 groups and 8 in the last 4. DCA was administered in the form of two 35 mg. pellets implantated subcutaneously at the time of uninephrectomy. Sandsotene,[3] Phenergan[3] and chlorpromazine[3] were given in daily doses of 2, 5 and 1 mg. respectively. Preliminary experiments had shown that more than 1 mg. of Chlorpromazine made the rats lethargic and drowsy The daily doses of these 3 drugs were administered in 2 subcutaneous injections of 0.1 cc. each. All the rats were fed Purina Fox chow and given 1% saline to drink. They were placed in individual metabolism cages for 3 days each week during which urine flow was measured. Blood pressure was taken once or twice a week by tail sphygmography (del Greco, Olmsted, Masson and Corcoran, 1953). The experiment was terminated on the 25th day; blood was drawn from the carotid artery for determination of blood urea nitrogen and serum chlorides. After macroscopic examination, heart, kidney and adrenals were fixed in Zenker's fluid; they were weighed, sectioned and stained with hematoxylin-eosin and Periodic acid-fuchsin stain. Pieces of the mesentery were similarly examined.

RESULTS

1. Physiologic changes (Table 1)

Growth, measured as weight gain, was inhibited in various degrees in all the experimental groups, except the one receiving Sandostene alone. Animals given DCA alone or in combination had edema and serous effusions which contributed to some of their body weight.

Scattered individuals of the control group and of groups not receiving DCA manifested arterial pressures of 150–180 mm. Hg throughout the experiment. At autopsy these animals showed mild hydronephrosis of the remaining kidney. This post-uninephrectomy hypertension is similar to, although not as common and severe as that reported by Grollman and Halpert (1949) in rats of the Evans-McCollum strain. In the DCA treated groups (II, VI to VIII), all the rats showed similar degrees of hypertension by about the 15th day. The means of the blood pressure levels of the animals given DCA show that Sandostene, Phenergan and chlorpromazine had no inhibitory effect on DCA-hypertension. The means and ranges of pressures in the groups not given DCA show that these 3 drugs did not influence postnephrectomy hydronephrotic hypertension either.

Urine flow, which in these experiments reflected fluid intake, was not

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[3] We are indebted to the Smith, Kline & French Laboratories, Philadelphia, Pa., for the chlorpromazine, the Wyeth Laboratories, Philadelphia, Pa., for the Phenergan and Sandoz Pharmaceuticals, Hanover, N. J., for the Sandostene.

definitely altered from the control levels by Phenergan, Sandostene or chlorpromazine. It was greatly increased in the group given DCA alone, and somewhat depressed below this level when DCA was given in combination with the three drugs. The significance of these differences was obscured by large individual variations in daily urine output. BUN and serum chloride concentrations were alike in all groups (ranges respectively 13 to 23 mg. per 100 ml. and 101 to 117 mEq. per liter).

2. Morphological changes.

At autopsy no macroscopic pathological changes were found in rats not given DCA with the exceptions, noted above, of slight hydronephrosis in 5 rats which had been moderately hypertensive. By contrast, the rats

TABLE 1. EFFECTS OF SANDOSTENE, PHENERGAN AND CHLORPROMAZINE OF BLOOD PRESSURE AND URINE FLOW IN DCA-TREATED RATS

Group	No. of rats	Treatment	Body weight in gm.		Blood pressure in mm. Hg on day			Urine flow in cc. per 24 hrs.	
			I.*	F.*	7	15	25	I.*	F.*
I	6	None	115 (100–130)	222 (211–241)	144 (134–156)	148 (125–160)	147 (125–160)	48 (35–60)	62 (49–72)
II	6	DCA	118 (100–135)	170 (155–183)	133 (108–185)	172 (150–185)	197 (160–230)	77 (39–111)	172 (164–183)
III	8	Sandostene	116 (102–130)	210 (189–235)	119 (102–133)	143 (120–170)	130 (115–150)	45 (39–58)	31 (15–45)
IV	8	Phenergan	115 (100–135)	150 (130–190)	131 (111–153)	151 (136–180)	146 (130–155)	57 (31–100)	60 (50–75)
V	6	Chlorpromazine	117 (100–135)	191 (165–210)	123 (113–142)	137 (110–180)	143 (130–175)	38 (24–55)	72 (35–110)
VI	6	Sandostene+DCA	119 (102–130)	167 (149–187)	137 (114–160)	190 (150–230)	216 (180–250)	73 (62–91)	106 (85–160)
VII	8	Phenergan+DCA	118 (100–135)	157 (122–180)	138 (115–153)	182 (135–225)	194 (155–250)	88 (80–121)	118 (108–128)

* I.=Initial; F.=Final.

receiving DCA (Groups II, VI to VIII) showed subcutaneous edema, fluid accumulation in the peritoneal and pleural cavities, hemorrhages and focal discoloration in the heart, granularity of the kidney and nodularities of the mesenteric arteries.

The means of the weights of kidneys and hearts (expressed in grams per 100 gm. of body weight) are given in Table 2. Those groups not given DCA, showed substantially the same organ weights as the control group. Those given DCA alone or in combination showed significantly higher weights than controls; there was no indication that the effects of DCA were modified by treatment with the drugs. The weights of the adrenals were not altered except that, as expected, they were slightly decreased by DCA treatment.

Histological examination confirmed and extended the macroscopic findings reported above; the characteristics of hypertensive vascular disease were seen only in the DCA-treated groups. The incidence of the lesions is

TABLE 2. MORPHOLOGICAL EFFECTS OF SANDOSTENE, PHENERGAN AND CHLORPROMAZINE IN DCA-TREATED RATS

Treatment	Kidney weight*	Heart weight*	Proportion of rats with vascular lesions in:	
			Heart	Kidney
None	0.68 0.62–0.74	0.29 0.26–0.31	0	0
DCA	1.07 1.01–1.12	0.41 0.36–0.45	0.7	0.5
Sandostene	0.63 0.60–0.67	0.27 0.25–0.31	0	0
Phenergan	0.65 0.56–0.70	0.29 0.27–0.30	0	0
Chlorpromazine	0.64 0.55–0.75	0.29 0.26–0.37	0	0
Sandostene + DCA	0.95 0.88–1.05	0.36 0.33–0.39	0.7	0.9
Phenergan + DCA	0.90 0.82–1.00	0.38 0.34–0.44	0.4	0.3
Chlorpromazine + DCA	0.91 0.77–1.09	0.41 0.36–0.44	0.7	0.5

* In grams per 100 gr. body weight.

given in the last 2 columns of Table 2 in which the number of rats in each group showing major vascular lesions is recorded as a fraction of the total number in each group. By major vascular lesions is meant those showing cellular infiltrates and intramural fibrinoid necrosis. The data demonstrate that chlorpromazine and Sandostene had no effect on the incidence of lesions. In the Phenergan-treated group (Group VII) the pathological changes were slightly less severe. We doubt that the difference is significant since these data are based upon histologic examination of no more than 2 to 4 sections from each organ.

DISCUSSION

The data presented indicate that the two antihistaminic drugs, in the doses used, and the chemically related compound, chlorpromazine, have no effect on the evolution of DCA-salt hypertension or hypertensive disease in the rat. In these experiments, the doses of Phenergan and Sandostene would have been sufficient to inhibit in the rat the edematous response to egg-white or dextran (Halpern 1949; Rothlin and Cerletti, 1953). Hence, inhibition of the vascular effects of free histamine, with whatever changes in vascular permeability may result therefrom, does not seem to have any influence on DCA-salt hypertension and hypertensive disease. The experiments do not exclude, however, the possibility that some histaminic mechanism may participate in the genesis of the

lesions, since peripherally released histamine is often not antagonized by antihistaminics (Goodman and Gilman, 1955).

The contrast between our observations and those of Zacco and Ciaca (1950), Guillemin and Fortier (1951) and of Rothlin and Cerletti (1954) demands some explanation. In this connection, it may be relevant that Braun-Menendez (1952) observed that large, intraperitoneal doses of Phenergan inhibited the appetite of DCA-treated rats for salt solution. DCA contributes to the genesis of hypertension and hypertensive disease in salt-treated rats primarily by its effect on salt intake and turnover. Hence, inhibition of salt intake as such would result in inhibition or amelioration of DCA-hypertension and DCA-induced lesions (Selye *et al.*, 1949). Unfortunately, Zacco and Ciaca (1950) do not record the effects of the drugs they tested on fluid (1% NaCl) intake and output and no data are available on the effect of Sandostene on the intake of 2% NaCl. Guillemin and Fortier (1951) and Guillemin (1951) noted that the 1% NaCl intake of their DCA-treated rats was "markedly reduced"; this seems to have suppressed, at least temporarily, the manifestations of the disease. In our experiments, Phenergan, Sandostene and chlorpromazine each caused moderate reduction in 1% saline intake, and, as we have shown, this degree of reduction was evidently insufficient to inhibit the manifestations of DCA-disease in the presence of DCA overdosage.

The observations described above, and also those of Braun-Menendez (1952) on the effects of various hormones and drugs on salt appetite emphasize the need for close supervision of salt intake in the evaluation of the antihypertensive effects of drugs in rats treated with salt solutions, with or without DCA supplements.

SUMMARY AND CONCLUSIONS

1. Phenergan, Sandostene and chlorpromazine, three related compounds of which the first two are antihistaminic, did not protect uninephrectomized rats against hypertension and the cardiovascular lesions consequent on treatment with DCA and salt.

2. Prior observations had indicated that antihistaminic drugs had a protective effect in such animals which was interpreted as a reflection of their effect on vascular permeability. The present data suggest that the effects of the antihistaminics and of chlorpromazine on salt intake may explain the discrepancy, so that apparent protection would be observed whenever the drugs induced a sufficient salt anorexia.

3. The experiments emphasize the importance or rigorous control of NaCl intake in therapeutic studies in DCA-salt hypertension.

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REFERENCES

BRAUN-MENENDEZ, E.: *Rev. de la Soc. Arg. de Biologia* **28**: 23. 1952.
DEL GRECO, F., OLMSTED, F., MASSON, G. M. C. AND CORCORAN, A. C.: *J. Lab. Clin. Med.* **41**: 729. 1953.
GOODMAN, L. AND GILMAN, A.: The Pharmacological Basis of Therapeutics. MacMillan Co., New York, 1955.
GROLLMAN, A. AND HALPERT, B.: *Proc. Soc. Exp. Biol. & Med.* **71**: 394. 1949.
GUILLEMIN, R.: *Semaine d. Hop.* Paris **27**: 18. 1951.
GUILLEMIN, R. AND FORTIER, C.: *Endocrinology* **48**: 617. 1951.
HALPERN, B. M.: *Presse Med.*, **57**: 949. 1949.
MASSON, G. M. C., CORCORAN, A. A. AND PAGE, I. H.: *J. Lab. & Clin. Med.* **34**: 1416. 1949.
ROTHLIN, E. AND CERLETTI, A.: *Int. Arch. Allergy* **4**: 1953.
ROTHLIN, E. AND CERLETTI, A.: Personal Communication.
SELYE, H.: *Canad. Med. Assn. J.* **47**: 515. 1942.
SELYE, H., STONE, H., TIMIRAS, P. S., AND SCHAFFENBURG, C.: *Am. Heart J.* **37**: 1009. 1949.
TRIPOD, J. AND MEIER, R.: *Arch. int. pharmacodyn.* **89**: 311. 1952.
ZACCO, M. AND CIASCA, G.: *Reumatismo* **2**: 1. 1950.

THE EFFECTS OF LOW PRESSURE ON HEMOPOIESIS IN THE ADRENALECTOMIZED RAT[1]

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IN VIEW of the steadily increasing emphasis being placed on the adrenal gland as a regulator of many phases of body metabolism, a detailed correlation of the peripheral blood changes to the bone marrow cytology in the adrenal insufficient animal, with and without replacement therapy, was made in an effort to determine the mechanisms underlying the influence of adrenal cortical principles upon the blood elements (Gordon and Piliero, 1950; Piliero, Landau and Gordon, 1950; Gordon, Piliero and Landau, 1951).

A more sensitive technique has been utilized for determining a possible relation of the adrenal to hemopoiesis through subjecting adrenalectomized and sham operated controls to an erythropoietic stress, namely lowered barometric pressures. The results of one phase of this study will be described in the present paper.

MATERIALS AND METHODS

Young adult female rats of a hardy, closely inbred strain and weighing 160–200 gm. were employed. All animals were fed a standard laboratory ration and kept at temperatures of 70–80 degrees F. The adrenalectomized rats were maintained on 1% NaCl given as drinking water.

Peripheral blood from the tail was employed for all procedures. Red and white cell counts were made in duplicate; the former were required to agree to within $\pm 4\%$ and the latter to within $\pm 10\%$. Differential counts were made from smears stained with Wright's stain. Eosinophiles were estimated by the method of Randolph (1949). Reticulocyte counts were made from wet mounts of whole blood suspended in 2% sodium citrate solution and stained with brilliant cresyl blue. One thousand cells were counted and the reticulocytes expressed as a percentage of these. Hemoglobin concentrations were determined by the acid hematin method with a photoelectric colorimeter. The method of Straus and Faigen (1946) was used to ascertain red cell fragilities in hypotonic saline. Sedimentation rates, measured as mm. fall per hour, were determined by the micromethod of Landau (1933). Duplicate hematocrit determinations from heparinized blood, spun at 6,000 r.p.m. for 15 minutes, were made by a capillary micromethod and were required to agree to within ± 1 mm. Control hemotologic determinations were carried out in all animals before experimental treatment was begun. The experimental groups were established as follows:

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Experiment I. Adrenalectomized and control animals following 14 to 21 days of exposure. Thirty adrenalectomized and 30 sham-adrenalectomized control animals operated 3 weeks previously were subjected to lowered barometric pressures in a specially constructed low pressure chamber. The animals received four hour exposures to pressures of 310.8 mm. Hg (equivalent to an altitude of 20,000 feet), every second day for 14 to 21 days. Hematologic examinations were made at weekly intervals.

Experiment II. Long term adrenalectomized and control animals following 14 to 21 days of exposure. Twenty adrenalectomized and 20 sham-adrenalectomized control animals operated 14 weeks previously were subjected to the same experimental procedures as in Experiment I.

At the termination of the observation periods, all rats were anesthetized lightly with ether and exsanguinated by cardiac puncture. The blood was allowed to clot at room temperature, stored in a refrigerator for 12 hours and the serum collected. The left femur was dissected, cracked open and the bone marrow removed and placed in a watch glass containing homologous serum obtained from previously killed rats. The marrow was prepared in suspension form by drawing it up and down gently in the serum with a glass pipette. Smears were made and fixed immediately in absolute methanol for 2 minutes and treated with May Grünwald stain. For each animal, 1,000 nucleated cells were counted for determination of the myelograms. The right femur was dissected, fixed in Helly's fluid, decalcified by a formic acid-ion exchange resin preparation (Win 3,000), sectioned serially in paraffin at a thickness of 5μ and stained with hematoxylin and eosin.

Weekly body weight measurements were made for all animals.

RESULTS[2]

Experiment I

A. Peripheral blood

The effects of 21 days of lowered barometric pressure upon the peripheral blood picture of adrenalectomized and control rats are shown in Tables 1 and 2. It will be noted that this erythropoietic stress results in red cell, hemoglobin, hematocrit and reticulocyte increases which are similar in the adrenalectomized and in the control group of animals. Following the cessation of the stimulus, red cell, hemoglobin, hematocrit and reticulocyte values tend to return to normal levels at approximately the same rate in the two groups of rats. Sedimentation rates and total white cell counts in both groups are uneffected by the reduced pressures. Likewise, differential white cell determinations show no consistent trends in either group. The application of lowered barometric pressures results in marked decreases in the red cell fragility values in both the control and adrenalectomized groups of animals. Interruption of the stimulus is followed by a tendency of the fragility values to be restored to pre-pressure levels.

The color index, volume index, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration

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[2] All statements made in this section regarding differences among the means of the different groups of animals compared are based on statistical analyses for probability values, derived from the distribution of Fisher's t. P values of <0.05 have been considered significant.

TABLE 1. EFFECTS OF LOW PRESSURE UPON ERYTHROCYTIC VALUES OF ADRENALECTOMIZED AND SHAM-ADRENALECTOMIZED RATS

(Means ± standard errors)

			RBC, million/mm.	Hemoglobin, gm. %	Reticulocyte, %	RBC frag., %	Hematocrit. %
SERIES 1							
Controls							
Exposed	initial	(30)[1]	8.24±0.24	16.65±0.45	1.6±0.22	90.4±3.00	50.4±1.6
	7 days	(30)	9.00±0.11	18.03±0.60	2.9±0.17	60.9±1.92	55.0±1.1
	14 days	(30)	9.39±0.34	18.24±0.39	3.5±0.28	70.1±1.87	55.4±1.7
	21 days	(30)	9.64±0.30	19.49±0.49	3.9±0.40	40.4±2.29	57.0±1.9
Rest Period	7 days	(15)	9.10±0.20	18.10±0.55	1.9±0.38	60.8±3.11	54.9±1.4
	14 days	(15)	8.54±0.26	17.42±0.38	1.4±0.27	71.1±2.75	52.1±1.7
	21 days	(15)	8.27±0.11	16.70±0.51	1.6±0.45	84.6±3.21	51.5±1.6
ADX							
Exposed	initial	(30)	7.89±0.22	16.27±0.35	1.4±0.41	82.1±2.17	50.1±1.1
	7 days	(30)	8.95±0.19	17.97±0.61	3.0±0.20	64.8±3.04	54.7±1.8
	14 days	(30)	9.37±0.32	18.24±0.44	3.2±0.29	49.9±1.99	56.1±1.0
	21 days	(30)	9.79±0.20	19.64±0.49	3.7±0.44	35.4±2.88	57.9±1.6
Rest Period	7 days	(15)	9.16±0.33	18.10±0.33	1.1±0.33	48.7±2.41	56.0±1.6
	14 days	(15)	8.89±0.17	17.75±0.29	1.2±0.28	69.4±2.07	54.9±1.8
	21 days	(15)	8.11±0.14	16.42±0.41	1.0±0.31	84.5±3.11	51.4±1.2
SERIES 2							
Controls							
Exposed	initial	(20)	8.36±0.24	16.71±0.59	1.6±0.29	94.3±2.87	50.5±1.4
	7 days	(20)	9.00±0.31	18.10±0.40	3.2±0.18	71.4±2.27	52.4±1.8
	14 days	(20)	10.04±0.33	19.84±0.39	3.9±0.41	69.7±3.11	57.9±1.5
ADX							
Exposed	initial	(20)	8.44±0.29	17.23±0.45	1.8±0.39	82.3±2.20	49.7±1.7
	7 days	(20)	9.94±0.21	19.59±0.50	3.8±0.22	39.7±2.96	54.8±1.2
	14 days	(20)	10.24±0.34	19.88±0.34	4.1±0.45	41.5±1.90	58.9±1.6

[1] Number of rats.

values of the control and adrenalectomized rats are unaffected by lowered barometric pressures.

B. Bone marrow studies

Observation of Table 3 reveals similar changes in the marrow cellular components of the normal and adrenelctomized rats subjected to the lowered barometric pressures. However, following a 3 week rest period subsequent to the last day of exposure the marrows of adrenalectomized animals possess greater numbers of total neutrophiles and smaller numbers of nucleated erythroid cells than the similarly sham-treated controls. The increase in neutrophiles is due largely to greater numbers of metamyelocytes and segmenters in the marrow smears of the adrenalectomized rats. Control rats which received a 3 week rest period reveal significant decreases in the nucleated erythroid cells and increases in the mature neutrophilic elements compared with rats studied immediately after pressure subjection.

C. Organ weights

Table 4 indicates that the low pressure stimulus employed is of sufficient intensity to increase significantly the adrenal weights of the female rat. Of especial interest is the finding that the spleen and thymus weights are greater in the exposed adrenalectomized than in the exposed control group.

TABLE 2. EFFECTS OF LOW PRESSURES UPON DIFFERENTIAL WHITE CELL COUNTS OF ADRENALECTOMIZED AND SHAM-ADRENALECTOMIZED RATS

(Means ± standard errors)

			Total WBC thousand, mm.³	Eosinophiles, mm.³	Lymphocytes, %	Neutrophiles, %
SERIES 1						
Controls						
Exposed	initial	(30)[1]	12.9 ± 1.7	377.5 ± 17.7	80.6 ± 2.2	17.0 ± 4.4
	7 days	(30)	13.3 ± 2.8	222.4 ± 23.4	77.3 ± 3.7	19.5 ± 2.9
	14 days	(30)	11.9 ± 1.8	259.4 ± 18.0	79.4 ± 4.1	17.8 ± 3.0
	21 days	(30)	11.9 ± 1.1	370.5 ± 20.1	78.4 ± 2.1	18.7 ± 4.5
Rest Period	7 days	(15)	11.0 ± 2.4	225.4 ± 40.0	77.2 ± 3.4	18.7 ± 2.2
	14 days	(15)	11.8 ± 2.8	365.9 ± 31.1	77.5 ± 4.5	18.6 ± 3.1
	21 days	(15)	11.5 ± 2.5	300.1 ± 27.7	76.9 ± 2.9	19.5 ± 4.6
ADX						
Exposed	initial	(30)	12.7 ± 1.4	277.5 ± 45.1	85.4 ± 2.4	12.7 ± 2.1
	7 days	(30)	13.2 ± 2.1	344.1 ± 24.7	82.1 ± 4.0	13.1 ± 1.9
	14 days	(30)	12.4 ± 2.9	288.4 ± 40.4	82.4 ± 4.1	14.9 ± 4.0
	21 days	(30)	12.9 ± 1.5	324.1 ± 45.5	80.4 ± 3.2	16.2 ± 5.1
Rest Period	7 days	(15)	12.0 ± 2.0	270.4 ± 25.9	80.5 ± 3.8	16.4 ± 1.9
	14 days	(15)	11.8 ± 2.8	294.1 ± 28.7	81.1 ± 4.1	16.7 ± 2.3
	21 days	(15)	11.4 ± 1.8	310.4 ± 29.4	79.8 ± 5.0	17.0 ± 3.0
SERIES 2						
Controls						
Exposed	initial	(20)	11.7 ± 1.4	188.7 ± 28.4	79.0 ± 2.8	18.1 ± 4.6
	7 days	(20)	12.0 ± 1.6	301.7 ± 20.1	83.1 ± 3.9	15.0 ± 2.9
	14 days	(20)	10.9 ± 2.4	222.0 ± 18.4	82.7 ± 2.7	16.2 ± 3.2
ADX						
Exposed	initial	(20)	13.5 ± 2.7	471.5 ± 17.2	83.0 ± 2.7	15.0 ± 3.0
	7 days	(20)	14.2 ± 2.0	310.8 ± 21.5	81.0 ± 3.4	17.0 ± 3.2
	14 days	(20)	13.9 ± 2.4	577.2 ± 19.2	76.0 ± 4.2	22.0 ± 2.7

[1] Number of rats.

Experiment II

A. Peripheral blood

Tables 1 & 2 indicate that the effects of a 14 day low pressure exposure period in long-term adrenalectomized rats are similar to those experienced in the short-term groups.

B. Bone marrow studies

Table 3 reveals that, as for the 21 day exposed short-term adrenalectomized rats, the 14 day exposure results in changes which are no different from those seen in the controls.

C. Organ weights

Observation of Table 4 reveals that the effects of low pressure upon the various organ weights studied in long-term adrenalectomized and sham operated animals are similar to those noted in the short-term animals.

TABLE 3. EFFECTS OF LOW PRESSURES ON BONE MARROW CELLULAR PERCENTAGES IN ADRENALECTOMIZED AND SHAM-ADRENALECTOMIZED RATS

(Means ± standard errors)

		Blasts	Neutrophiles[1]		Eosinophiles	Lymphocytes	Nucleated Erythrocytes
			Young	Mature			
SERIES 1							
Controls							
Exposed	21 days (15)[2]	0.2±0.2	6.5±0.8	26.6±1.5	5.0±0.8	9.6±2.2	50.2±2.5
Rest Period	21 days (15)	0.4±0.1	6.1±0.5	34.0±1.1	6.6±0.9	10.4±2.1	41.0±2.2
ADX							
Exposed	21 days (15)	0.6±0.1	5.8±0.7	24.7±1.7	5.2±0.7	12.3±1.9	48.0±1.8
Rest Period	21 days (15)	0.8±0.1	5.5±0.9	39.4±1.4	6.7±0.8	11.4±2.8	32.5±2.1
SERIES 2							
Controls							
Exposed	14 days (20)	0.2±0.2	6.0±0.7	26.5±1.2	5.7±0.5	10.2±2.3	47.4±1.4
ADX							
Exposed	14 days (20)	0.5±0.4	6.2±0.5	25.8±1.5	7.7±0.6	12.2±1.8	46.6±1.8
Controls[3]							
Post Op.	21 days (10)	0.2±0.1	5.1±0.7	35.1±1.0	7.8±0.8	13.2±2.1	38.5±1.9
ADX[3]							
Post Op.	21 days (10)	0.1±0.1	6.0±0.6	37.0±0.9	5.2±0.7	12.3±1.9	38.0±1.6

[1] Young neutrophiles included promyelocytes and myelocytes; mature neutrophiles include metamyelocytes and segmented forms.
[2] Number of rats.
[3] Data from paper in press (Acta Haematologica).

DISCUSSION

That the hemoglobin and red blood cell concentrations are greater in individuals at high altitude than at sea level is well known (Bert, 1882; Douglas, Haldane, Henderson and Schneider, 1913; Gordon and Kleinberg, 1937; Hurtado, Merino and Delgado, 1945; Schneider, 1921). Hematocrits have also been shown to increase in proportion to the number of exposures (Altland and Highman, 1951).

TABLE 4. EFFECTS OF LOW PRESSURES UPON THE BODY, ADRENAL GLANDS, SPLEEN AND THYMUS WEIGHTS OF ADRENALECTOMIZED AND SHAM-ADRENALECTOMIZED RATS

(Means ± standard errors)

		Body wt., gm.		Spleen wt., mg.	Thymus wt., mg.	Adrenal wt., mg.
		Initial	Final			
SERIES 1						
Controls						
Exposed	21 days (15)[1]	165.7±6.2	181.0±6.8	685.1±38.1	187.7±19.9	56.4±4.0
Rest Period	21 days (15)	162.3±6.4	184.7±7.6	615.6±71.9	187.0±21.1	54.3±2.5
ADX						
Exposed	21 days (15)	180.5±5.8	200.7±6.4	779.6±29.9	251.4±25.0	
Rest Period	21 days (15)	186.0±6.7	215.1±5.4	871.9±41.4	260.0±24.9	
SERIES 2						
Controls						
Exposed	14 days (20)	185.1±4.7	198.3±4.4	672.1±29.1	180.0± 7.0	57.6±3.1
ADX						
Exposed	14 days (20)	197.3±6.3	211.9±5.9	981.3±71.2	274.1±20.1	
Controls[2]						
Post Op.	21 days (10)	162.4±5.1	182.5±5.4	579.1±78.1	200.0±15.9	47.2±3.0
ADX[2]						
Post Op.	21 days (10)	166.7±6.5	183.1±7.7	497.4±50.6	165.7±24.9	

[1] Number of rats.
[2] Data from paper in press (Acta Haematologica).

Gordon, Tornetta, D'Angelo and Charipper (1943) have demonstrated that anoxia, the generally acknowledged "fundamental erythrocytogenic stimulus" results in morphologic and physiologic alterations in various endocrine glands including the pituitary, adrenal, thyroid and gonads. The suggestion has been made (Gordon and Charipper, 1947) that anoxic stimuli may affect hemopoiesis as a result of operating through hormonal mechanisms. It seems clear, however, from the present work that the adrenal is not of importance in this connection since hypoxia induced by lowered pressures invokes peripheral and bone marrow erythroid responses of approximately the same magnitude in the adrenalectomized and control animals. Apparently, despite the anemic state of the adrenalectomized rat, sufficient erythroid elements can be mobilized to match the response of the control animal to erythropoietic stress. The bone marrow studies, however, indicate that a debt is eventually incurred since, by 3 weeks following the cessation of the low pressure stimulus, the nucleated erythroid cell numbers of the adrenalectomized rat have fallen significantly lower than those of the controls. Further investigation is being conducted in the clarification of this interesting problem by a study of the effects of resubjecting the adrenalectomized animals at this time to reduced pressures.

Of especial significance are the results of Feigin and Gordon (1950) which demonstrate that the hypophysectomized rat can respond with increased erythropoiesis to an extent similar to that experienced by intact controls when exposed to low pressures corresponding to 321 mm. Hg. Therefore, as for the adrenal, it seems that the presence of the pituitary is not essential for the erythropoietic response to anoxia. These findings and the results of our present study do not preclude the possibility that the endocrine system is involved in the hemopoietic response of the intact animal to lowered pressures. It has been suggested by Gordon (1954) that the endocrine glands may function as a buffer mechanism, serving to adjust the quality and intensity of the erythropoietic reaction in response to the requirements for oxygen by the body cells.

SUMMARY

1. Red blood cell counts, hemoglobin, hematocrit and reticulocyte values in the adrenalectomized and control groups respond to lowered barometric pressures with approixmately equal intensity.

2. No differences are observable in the bone marrow response of the control and adrenalectomized rats to 21 days of low pressure exposure. Following a three week rest period, greater numbers of granulocytes and decreases in the numbers of nucleated erythroid cells, with a resulting lowered erythroid-myeloid cell ratio, become apparent in the marrows of the adrenalectomized rats.

3. It is concluded that although adrenalectomy in the rat results in alterations in the red cell production and/or destruction mechanisms, the

adrenal is not essential for the response to the erythropoietic stress imposed by lowered barometric pressures.

REFERENCES

ALTLAND, P.D. AND B. HIGHMAN: *Am. J. Physiol.* **167:** 261. 1951.
BERT, P.: *Compt rend. Acad. Sci.* **94:** 805. 1882.
DOUGLAS, C. G., J. S. HALDANE, Y. HENDERSON AND E. C. SCHNEIDER: *Phil. Tr. Roy. Soc.*, London **203B:** 185. 1913.
FEIGIN, W. M. AND A. S. GORDON: *Endocrinology* **47:** 364. 1950.
GORDON, A. S.: *Rec. Progr. Hormone Res.* **10:** 339. 1954.
GORDON, A. S. AND H. A. CHARIPPER: *Ann. N. Y. Acad. Sci.* **48:** 615. 1947.
GORDON, A. S. AND W. KLEINBERG: *Proc. Soc. Exp. Biol. and Med.* **37:** 507. 1937.
GORDON, A. S. AND S. J. PILIERO: *Fed. Proc.* **9:** 49. 1950.
GORDON, A. S., S. J. PILIERO AND D. LANDAU: *Endocrinology* **49:** 497. 1951.
GORDON, A. S., F. J. TORNETTA, S. A. D'ANGELO AND H. A. CHARIPPER: *Endocrinology* **33:** 366. 1943.
HURTADO, A., C. MERINO AND E. DELGADO: *Arch. Int. Med.* **75:** 284. 1945.
LANDAU, A.: *Am. J. Diseases of Children* **45:** 691. 1933.
PILIERO, S. J., D. LANDAU AND A. S. GORDON: *Science* **112:** 559. 1950.
RANDOLPH, T. G.: *J. Lab. and Clin. Med.* **34:** 1696. 1949.
SCHNEIDER, E. C.: *Physiol. Rev.* **1:** 631. 1921.
STRAUS, R. AND R. FAIGEN: *Am. J. Clin. Path.* **16:** 437. 1946.

MULTIPLE RESPONSES IN HEPATIC GLYCOGEN DEPOSITION ASSAYS USING INTACT MICE

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INTRODUCTION

IN THE biological assay of glucocorticoids, such as cortisone, it may happen that increasing doses of the drug result not only in a graded increase in deposited liver glycogen, but also in a graded inhibition of growth as measured by the final (pre-autopsy) body weight of the animal. A problem exists as to combining these two measurements, final body weights and total glycogen, into a single meaningful response. A time-honored solution is to divide the total glycogen by the final body weight so as to obtain a "percentage liver glycogen." Although this procedure will, in valid assays, yield unbiased estimates of relative potency, it has the disadvantage of not always providing potency estimates of the highest precision obtainable from the data. This is readily seen from consideration of the ratio

$$U = \frac{(\text{Total Liver Glycogen})^{\alpha_2}}{(\text{Final Body Weight})^{\alpha_1}}$$

The use of the glycogen/body weight ratio, in essence, invokes the arbitrary assumption that $\alpha_1 = \alpha_2 = 1$, an assumption which may deviate markedly from the truth.

When the investigator possesses assay data consisting of final body weight and total liver glycogen, both of which are affected by the drug, he is dealing with a problem in discriminant function analysis. This type of problem is to be clearly distinguished from that which obtains when the animals are weighed prior to injection of the drug; then the body weights are not affected by the drug.

Discriminant function analysis was first developed by Fisher (1936), and has since received widepsread application in a number of fields such as osteometry, psychometry, personnel counseling, etc. Its application to bioassay problems was pioneered by Bliss and Bartels (1946). Finney (1952) has given an example of its use in bioassay. He was, however, forced to use an artificial example owing to the paucity of genuine data on assays using several response measurements. More recently, Rao (1954) has pro-

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vided the derivation of valid fiducial limits to the potency, using for his example Finney's artificial data, again because of the dearth of genuine material.[1]

In a parallel line assay the inherent precision of the procedure is measured by $S/b=\lambda$ (Ott, 1952), the standard error of a log dose estimated from a single response assuming that the dose response curve is known exactly. Discriminant analysis in a bioassay has for its objective the establishment of a linear composite function of the observed responses whose parameters are so chosen that λ is reduced to a minimum.

The purpose of this paper is: (1) To illustrate the technique by its application to a genuine bioassay of a glucocorticoid by hepatic glycogen deposition. (2) To determine the ratio of α_2/α_1, which maximizes the precision of the assay. (3) To compare the estimate of potency and its fiducial limits obtained from the analysis with the estimate and limits obtained by use of the arbitrary ratio of $\alpha_2/\alpha_1=1$.

PRELIMINARY

A total of 54 intact male mice of the Carworth Farms CF1 strain was randomly assigned to 6 dose groups. The initial weights of the animals averaged approximately 18 gm.

The Reference Standard was hydrocortisone (as the free alcohol) with an assigned potency of 100%. Both the Standard and the sample of unknown potency submitted for assay were prepared in aqueous suspension in dilutions such that each injection dose was contained in 0.1 ml. The animals were subcutaneously injected once a day for the first three days, twice a day on the fourth day, and once on the fifth day.

During the course of injections the animals had free access to a diet of commercial mouse pellets and water until 14 hours prior to the last injection, when all food was withdrawn. Two hours after the last injection the animals were weighed, sacrificed, and their livers excised. Upon excision each liver was weighed and transferred to a test tube immersed in a dry ice-alcohol bath. Glycogen determinations were then performed using the procedure described by Kahan (1953).

Preliminary Calculations: The first step in the analysis is the estimation of the parameters α_2, α_1 in

$$U = \frac{(\text{Total Liver Glycogen (mg.)})^{\alpha_2}}{(\text{Final Body Weight (g./10)})^{\alpha_1}} \tag{1}$$

This ratio may be transformed by logarithms to the form

$$Y = \log_{10} U = \alpha_2 Y_2 - \alpha_1 Y_1 \tag{2}$$

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[1] A reader of the manuscript of this article has drawn the author's attention to two additional papers applying discriminant analysis to bioassay. These are:

Ipsen, J., "Appropriate Scores in Bioassays Using Death-Times and Survivor Symptoms." Abstract No. 305, *Biometrics*, **11**, 240.

Liddle, G. *et al.*, "Physiological Basis for a New Method of Assaying Aldosterone in Extracts of Human Urine." To be published *J. Clin. Investigation*. September, 1955.

Where

$$\begin{cases} Y_2 = 10 \log_{10} (\text{Total Hepatic Glycogen (mg.)}) \\ Y_1 = 10 \log_{10} (\text{Final Body Weight (gms.)}/10)) \end{cases} \tag{3}$$

These quantities will be found in Table 1 together with the coded values of x, the log dose. These are obtained by noting that the doses for both the Standard and Unknown are in geometric progression. The ratio between adjacent dose pairs is $\sqrt{10}$; consequently, the values of x are given by

$$x = \log_{\sqrt{10}} \frac{(\text{Dose (mg./mouse/injection)})}{(0.158)}$$

where 0.158 is the intermediate dose of steroid in mg./mouse/injection. The values of x so obtained are -1, 0 and $+1$. (See Table 1).

The next step is the computation of an analysis of dispersion (Rao, 1952, 1954). Formally, this is exactly the same operation as computing an analysis of covariance between Y_1 and Y_2 in a 6-point assay by the methods described by Finney (1952) and by Bliss (1951). The completed analysis is presented in Table 2 where the symbols SY_1^2, SY_1Y_2, and SY_2^2 refer to sums of squares and products about the general means.

TABLE 1. RESULTS OF BIOLOGICAL ASSAY

$Y_1 = 10 \log_{10}$ (Final Body Weight in units of 10 gms.)
$Y_2 = 10 \log_{10}$ (Total Hepatic Glycogen in mg.)

$$x = \log_{\sqrt{10}} \left(\frac{\text{Dose (mg./mouse/injection)}}{0.158} \right) = -1, 0 \text{ and } 1$$

Doses (mg./mouse/injection) = 0.05, 0.158 and 0.5

Drug	Value of x					
	-1		0		1	
	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2
Standard = S Hydrocortisone	3.0	11.2	2.2	4.8	1.8	15.4
	3.2	8.6	2.9	4.8	3.2	17.6
	3.4	13.8	2.9	17.3	2.5	5.6
	3.0	8.3	3.6	17.3	2.8	16.5
	2.9	9.9	3.0	19.6	2.7	20.0
	3.6	9.9	2.9	17.6	2.8	18.2
	3.1	7.6	3.0	16.0	2.8	17.8
	2.6	2.8	3.0	16.2	2.8	18.7
	3.1	12.2	3.0	16.1	2.8	18.2
Total	27.9	84.3	26.5	129.7	24.2	148.0
Unknown for assay = U	3.0	7.8	3.1	12.8	3.2	18.5
	2.4	9.0	3.2	12.8	2.8	14.0
	3.0	9.0	3.0	12.4	3.0	17.4
	3.0	9.4	3.3	16.4	3.0	16.4
	3.3	7.8	1.6	2.6	3.0	14.4
	3.1	13.3	2.6	9.5	2.6	13.8
	3.8	14.4	2.7	10.8	2.7	14.4
	2.8	9.4	2.6	15.3	2.3	12.2
	3.5	12.1	2.6	8.9	2.6	15.4
Total	27.9	92.2	24.7	101.5	25.2	136.5
Mean Responses:			$\bar{Y}_1u = 2.8814$		$\bar{Y}_2u = 12.2296$	
			$\bar{Y}_1s = 2.9111$		$\bar{Y}_2s = 13.4074$	

TABLE 2. ANALYSIS OF DISPERSION

Nature of variation	*df*	SY_1^2	SY_1Y_2	SY_2^2
Total	53	8.260	24.484	1061.522
Samples	1	0.012	0.471	18.727
Slope	1	1.138	−19.200	324.000
Non-parallelism	1	0.028	−0.539	10.454
Curvature	1	0.072	−0.036	0.018
Opp. Curvature	1	0.196	2.249	25.813
Error	48	6.814	41.539	682.510

		(1)	(2)	(3)	$\overset{\Delta}{(1)\cdot(3)-(2)^2}$
Error + Samples	49	6.826	42.010	701.237	3021.804
Error + Slope	49	7.952	22.339	1006.510	7504.736
Error + Non-parallelism	49	6.842	41.000	692.964	3060.260
Error + Curvature	49	6.886	41.503	682.528	2977.389
Error + Opp. Curvature	49	7.010	43.788	708.323	3047.955
Error	48	6.814	41.539	682.510	$2925.135 = \Delta_E$

Regression Coefficients

$SY_1x = -6.4$ $\quad -0.1778 = b_1$
$SY_2x = 108.0$ $\quad 3.000 = b_2$
$Sx^2 = 36.0$

TESTS FOR ASSAY VALIDITY

The entries in Table 2 are the basis of the assay validity tests which are made by computing the value of the variance ratio

$$F = \frac{n-p}{p}\left(\frac{\Delta}{\Delta_E} - 1\right) \tag{5}$$

with p and $(n-p)$ degrees of freedom (d.f.) for each of the orthogonal contrasts in Table 2 and where

n = Total d.f. for error + tested contrast = 49

p = Number of responses = 2

Δ/Δ_E = Ratio of Δ for error + tested contrast to Δ_E.

The results of these tests are shown in Table 3.

TABLE 3. SUMMARY OF VALIDITY TESTS

Contrast Tested	Δ/Δ_E	$F\left(=\frac{47}{2}\left(\frac{\Delta}{\Delta_E}-1\right)\right)$	P
Samples	1.033	0.78	>0.05
Slope	2.565	36.78	$\ll 0.001$
Non-parallelism	1.046	1.08	>0.05
Curvature	1.018	0.42	>0.05
Opp. Curvature	1.042	0.99	>0.05

The non-significance of the F-ratios for non-parallelism and for curvature together with the highly significant ratio for slope confirm the validity of the assay.

TESTS FOR ADDITIONAL INFORMATION

Before calculating the relative potency of the Unknown, it is desirable to determine whether the use of two measurements increases the quantity of information over the quantity provided by a single measurement considered alone. Considering the first measurement, Y_1—the value of Δ/Δ_E for Y_1 alone is $7.952 \div 6.814 = 1.167$ with an F-ratio of $(48/1)\ (0.167) = 8.02$ on 1 and 48 d.f. This is significant; hence, Y_1 provides information (non-zero regression of Y_1 on log dose) on potency. The additional information contributed by Y_2 is obtained from the ratio of the Δ/Δ_E values for Y_1 and Y_2 jointly and Y_1 alone; this is $2.565 \div 1.167 = 2.198$. The corresponding F-ratio is $(47)\ (2.198 - 1) = 56.31$ on 1 and 47 d.f. This is very highly significant and leads to the conclusion that the joint use of Y_1 and Y_2, rather than Y_1 alone, very significantly improves the assay precision. A similar test yields $F = 22.80$ (d.f. 1,48) for Y_2 alone, and $F = 34.73$ (d.f. 1,47) for the additional information from Y_1. These tests indicate the quantity of information provided by consideration of Y_1 or Y_2 alone is significantly less than the quantity yielded by the use of Y_1 and Y_2 jointly.

TEST OF THE ADEQUACY OF AN ARBITRARY FUNCTION

The statistical tests demonstrated in the previous section indicate that Y_1 and Y_2 should be considered jointly if the assay precision is to be maximized. The next question to be tackled is whether the arbitrary ratio of $\alpha_2 : \alpha_1 = 1$, implied in the use of the glycogen/body weight ratio, results in maximum discrimination between the dose groups.

Let the arbitrary linear function be

$$Y_0^* = (1)Y_2 + (-1)Y_1 \tag{6}$$

where Y_1 and Y_2 are defined in equation (3). The sum of squares for any contrast may be found by insertion of the appropriate terms from Table 2 into

$$SY_0^{*2} = \alpha_1^2 SY_1^2 + 2\alpha_1\alpha_2 SY_1Y_2 + \alpha_2^2 SY_2^2 \tag{7}$$

For the special case of $\alpha_2 = 1$, $\alpha_1 = -1$, we have

$$SY_0^{*2} \text{ for slope} = (-1)^2 1.138 + 2(-1)(1)(-19.200) + (1)^2 324.00 = 363.538$$

and for error

$$SY_0^{*2} = (-1)^2(6.814) + 2(-1)(1)(41.539) + (1)^2 682.51 = 606.246.$$

The ratio (Error+Regression)/(Error) for Y_0^* is

$$\left(1 + \frac{363.538}{606.246}\right) = 1.600$$

with an F-ratio of $(48/1)\ (0.600) = 28.80$ with 1 and 48 d.f. This is very highly significant.

The value of Δ/Δ_E for Y_1 and Y_2 jointly is 2.565 which exceeds the value for Y_0* by $2.565 \div 1.600 = 1.6031$. The corresponding F-ratio is $(47) \cdot (0.6031) = 28.34$ on 1 and 47 d.f. This latter value is very highly significant, and consequently it is concluded that the arbitary values of $\alpha_2 = 1$, $\alpha_1 = -1$ do not provide complete information on the potency.

DETERMINATION OF BEST LINEAR FUNCTION

The best values of α_1, α_2 are obtained by solving

$$\left.\begin{aligned} SY_1^2 a_1 + SY_1Y_2 a_2 &= b_1 \\ SY_1Y_2 a_1 + SY_2^2 a_2 &= b_1 \end{aligned}\right\} \tag{8}$$

where a_1, a_2 rather than α_1, α_2 are used to indicate the calculated values, and where SY_1^2, SY_1Y_2, and SY_2^2 are the error items in Table 2. The numerical equations are

$$\left.\begin{aligned} 6.814a_1 + \ 41.539a_2 &= -0.1778 \\ 41.539a_1 + 682.510a_2 &= \ \ 3.0000 \end{aligned}\right\} \tag{8a}$$

giving

$$\left.\begin{aligned} a_1 &= -0.08407 \sim -0.08 \\ a_2 &= \ \ 0.00951 \sim \ \ 0.01 \end{aligned}\right\} \tag{8b}$$

as the solution. Thus, the best linear function can be written

$$Y^* = 0.01Y_2 - 0.08Y_1$$

RELATIVE POTENCY AND FIDUCIAL LIMITS

Using the notation of indicating the Unknown and Standard preparations by the subscripts u and s and using M for log to the base $\sqrt{10}$ of the relative potency, the statistics

$$\left.\begin{aligned} T_1 &= (\bar{y}_{1u} - \bar{y}_{1s} - Mb_1) = -0.2970 + 0.1778M \\ T_2 &= (\bar{y}_{2u} - \bar{y}_{2s} - Mb_2) = -1.1778 - 3.0000M \\ \mu &= (1/n_1 + 1/n_2 + M^2/Sx^2) = 0.074074 + M^2/36 \end{aligned}\right\} \tag{9}$$

are formed. These are next inserted into the expression

$$SY_2^2T_1^2 - 2SY_1Y_2T_1T_2 + SY_1^2T_2^2 = \frac{2\mu}{(N_e - 1)} \cdot (F5\%\ \Delta_E) \tag{10}$$

where

N_e = error degrees of freedom = 48

$F5\%$ = the tabular 5% value of the F-ratio on 2 and $N_e - 1$ degrees of freedom (2 and 47)

Δ_E = Error determinant from Table 2 = 2925.135

Insertion of the appropriate values and collecting terms gives

$$M^2 + 0.439181\ \mathrm{M} - 0.192639 = 0$$

a quadratic in M with roots $M_1 = -0.710340$ and $M_2 = 0.271159$ as the 95% fiducial limits on $M = \log_{\sqrt{10}}$ (potency).

The limits on the potency are then

$$R_1\% = 100\%(\sqrt{10})^{-0.710340} = 44\%$$
$$R_2\% = 100\%(\sqrt{10})^{+0.271159} = 137\%$$

Finally, the point estimate of potency is obtained first by inserting

$$D_1 = \bar{y}_{1u} - \bar{y}_{1s}$$
$$D_2 = \bar{y}_{2u} - \bar{y}_{2s}$$

into the equation

$$1p = a_2 D_2 + a_1 D_1$$

where the coefficients a_1, a_2 are those calculated in equation (8b) and the y's are the mean responses given in Table 1.

The value of $1p$ so obtained is

$$1p = (0.00951)(-1.1778) + (-0.08407)(-0.02970) = -0.0087$$

Next, the regression coefficients b_1 and b_2 in Table 2 are inserted in the equation

$$1_1 = 0.00951 b_2 - 0.08407 b_1 = 0.0434$$

where, again, the coefficients are obtained from equation (8b). The ratio $1p/1_1$ leads to the point estimate of potency as follows:

$$\text{Relative Potency } R\% = 100\%(\sqrt{10})^{(-0.0087)/(0.0434)}$$
$$\text{or, } R\% = 79\%$$

These fiducial limits of 44% to 137% are 56% to 170% of the estimate. It is now of interest to compare these with the estimate of 66% with limits of 28% to 135% which are obtained by use of the arbitrary function, Y_0*. These latter limits are 42% to 205% of the estimate and are much wider than those obtained by the joint analysis.

DISCUSSION

It may perhaps be felt at this point that the foregoing analysis is somewhat akin to slaying a butterfly with a steam hammer, and it might be questioned whether the increase in precision is worth the extra labor incurred. Preliminary to answering this question, it is necessary to consider the increase in information resulting from the use of the optimum function, Y*, as compared with the arbitrary function, Y_0*. The relative gain in information yielded by the assay using the optimum function may be ap-

proximately estimated as the square of the ratio of the indices of precision.

The required indices of precision may be obtained from the formula

$$\lambda = \frac{(\log_{10}\sqrt{10})\sqrt{\alpha_1^2 Sy_1^2 + \alpha_2^2 Sy_2^2 + 2\alpha_1\alpha_2 Sy_1y_2}}{\sqrt{K}(\alpha_2 b_2 + \alpha_1 b_1)} \tag{11}$$

where

$K = 48$ for the arbitrary function and 47 for the optimum function

$\left.\begin{aligned}\alpha_1 &= -1\\ \alpha_2 &= 1\end{aligned}\right\}$ for the arbitrary function, Y_0^*

$\left.\begin{aligned}\alpha_1 &= a_1 = -0.08407\\ \alpha_2 &= a_2 = 0.00951\end{aligned}\right\}$ for the optimum function, Y^*

$b_1 = -0.1778$

$b_2 = 3.000$

Insertion of these quantities into equation (11) yields

$\lambda_0 = 0.5592$ for the arbitrary function

$\lambda = 0.3503$ for the optimum function

The relative gain in information is therefore

$$\text{Relative Information} = I_R = 100\%\left(\frac{0.5592}{0.3503}\right)^2 = 255\%$$

Thus, to obtain the same precision using the arbitrary function, Y_0^*, as was obtained by use of the optimum function, Y^*, a total of 138 mice would have been required instead of the 54 actually used. This reduction in assay cost is somewhat counterbalanced by the fact that the cost of the statistical analysis was increased about two-fold. It must, however, be borne in mind that any reduction in the number of animals required to attain a selected degree of precision is a corresponding saving in the number of determinations of reducing substances needed for each assay and also in the number of injections required. Another consideration which may be of importance is that, in situations where the substance for assay is in short supply, any reduction in the quantities needed would be a real advantage.

The conditions which appear to be generally employed in the performance of hepatic glycogen deposition assays are such that the glycogen response is well defined while the action of the drug on body weight is relatively small. In this situation the investigator either ignores the final body weight or computes the glycogen/body weight ratio. This, however, may not be the best policy; a more precise estimate of potency may possibly be obtained by adjusting assay conditions so that both the body weight and liver glycogen respond linearly to the drug. Should it then be found that the estimates of the parameters in a linear discriminant based on these two responses are reasonably stable in repeated assays, a suitable table calculated from the function could then be routinely employed.

SUMMARY

(1) A biological assay of a glucocorticoid, in which the drug affected glycogen and body weight, has been examined by means of multivariate analysis to determine the linear function which maximizes the assay precision.

(2) The index of precision using the optimum linear function was 0.35, while the index obtained by use of the logarithm of the glycogen/final body weight ratio was 0.56. The increase in information resulting from the use of the optimum function was approximately 155%.

(3) The linear discriminant found to maximize the precision of the assay was

$$Y^* = \log_{10}\left(\frac{\text{(Liver Glycogen (mg.))}}{\text{(Final Body Weight (deg.))}^8}\right).$$

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REFERENCES

Bliss, C. I., Bartels, B. L.: *Proceedings, Part II. Federation of American Societies for Experimental Biology* **5:** 167–168. 1946.

Bliss, C. I.: *Vitamin Methods*, Volume II, edited by P. Gyorgy. Academic Press Inc., New York. 1951.

Finney, D. J.: *Statistical Method in Biological Assay*. Charles Griffin and Co., London. 1952.

Fisher, R. A.: *Ann. Eugen.*, London. **7:** 179. 1936.

Kahan, J.: *Archives of Biochemistry and Biophysics*, **47:** 408. 1953.

Ott, W. H.: *Proceedings of the All Day Conference on Quality Control*, September 13, 1952.

Rao, C. R.: *Advanced Statistical Methods in Biometric Research*. John Wiley and Sons, New York, pp. 236–272. 1952.

Rao, C. R.: "Estimation of Relative Potency from Multiple Response Data." *Biometrics* **10:** 208. 1954.

THE METABOLISM OF RADIOESTRONE IN THE RAT

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DURING the period of twenty-five years since the isolation of the first crystalline estrogen many experiments on the metabolism of estrogens have been reported. Since a number of reviews (Doisy *et al.*, 1942; Heard, 1949; Lieberman and Teich, 1953) have considered this subject, only a few points which are closely relevant to the experiments reported in this paper will be mentioned. Most investigators studied the excretion of estrogens in urine, but the low recoveries (5–20%) of administered estrogenic activity led others to search for the unaccounted for estrogen. The recovery of some additional estrogenic activity in feces (Siebke and Schuschania, 1930; Levin, 1945; Dingemanse and Laqueur, 1937; Dorfman, 1937), led to the examination of bile (Longwell and McKee, 1942; Cantarow *et al.*, 1942; Pearlman *et al.*, 1948). However, the amounts detected in dog bile were still insufficient to account for the missing estrogen (Longwell and McKee, 1942; Pearlman *et al.*, 1948).

Since the earlier studies, which were based mainly on the recovery of estrogenic activity, accounted for only a small proportion of the original estrogen, the application of isotopic techniques offered great promise in searching for other metabolites. In pursuing this approach, studies were conducted with estrogens labeled with Br^{82} (Twombly *et al.*, 1948) and I^{131} (Albert *et al.*, 1949). Although these compounds possessed only low estrogenic activity, these investigations contributed useful information. Estrone sulfate-S^{35} was used but the results were equivocal due to rapid hydrolysis *in vivo* of the ester (Hanahan and Everett, 1950; Davis *et al.*, 1950). Pearlman's studies (1954) with 6,7-d_2-estrone acetate in pregnant women were limited to an investigation of the recovery and interconversion of the deuteroestrogens excreted in urine.

The synthesis of estrone-16-C^{14} by Heard and coworkers (cited by Leblond, 1952) provided an excellent compound for the metabolic study of estrone. This radioestrone has been studied *in vivo* in mice (Thompson and Heard, 1952; Budy, 1953), rabbits (Stimmel, 1954), humans (Beer *et*

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al., 1953; Beer and Gallagher, 1955) and the pregnant mare (Solomon and Heard, 1952). The present report concerns the excretion of C^{14} after administration of estrone-16-C^{14} to spayed rats with intact, cannulated or ligated bile ducts. Partial characterization of the radioactive metabolites in bile and urine is included.

EXPERIMENTAL

Estrone-16-C^{14}. As received, this compound[2] had a specific activity of 2.7 microcuries per mg. The melting point and biologic activity of this radiohormone were identical with those of estrone.

Radioassay. All samples were oxidized to CO_2, precipitated and counted as $BaCO_3$ under a thin window Geiger tube or in a gas flow counter. All counts were converted to disintegrations per minute after comparison with a standard sample of $BaC^{14}O_3$.

Bioassay. The Emmens intravaginal bioassay method (Emmens, 1941) was used. This delicate procedure was necessary because of the minute amounts of estrogenic compounds to be measured. In our hands 0.0003 μg. of estrone, 0.0005 μg. of estradiol-17β or 0.002 μg. of estriol gave positive smears in 50% of the animals used for assay. Bile from normal rats was fractioned and the respective fractions tested for their effect on the bioassay of estrone, estradiol-17β and estriol. Neither enhancement nor diminution of potency was observed.

Excretion of C^{14} in Urine, Feces and Bile. Adult, spayed female white rats from the St. Louis University Colony were employed. Estrone-16-C^{14} was administered either intramuscularly in dibutyl succinate or intragastrically in absolute ethanol or in a mixture of equal parts of water and 95% ethanol. Fifty μg. of estrone-16-C^{14} were administered in 0.25 cc. of solvent to each animal of the intragastric series, and 50 μg. in 0.20 cc. to each animal of the intramuscular series.

Each series consisted of three types of animals: control animals (NC), animals with doubly-ligated and severed bile ducts (DL), and animals with cannulated bile ducts (BF). Metal-tipped polyethylene tubing was inserted in the bile duct and the animal was placed in a restraining cage (Bollman, 1948). The excreta were collected for 5 days in the intramuscular series and for 4 days in the intragastric series.

An examination of Table 1 shows that the excretory pathways were similar after both methods of administration. In the control series about 20% of the C^{14} was recovered in the urine and 70% in the feces. The duct-ligated animals excreted radioactivity only in the urine, in which about 90% was recovered. In the duct-cannulated animals the bile proved to be almost the sole excretory pathway with only a slight amount of radioactivity detectable in the first 24-hour urine of all of the intragastric series. In this group, only one animal excreted C^{14} in the urine on the second day. One animal in the intramuscular series excreted only 1.1% of the C^{14} in the urine on the first day.

As depicted in Figures 1a and 1b the C^{14} was excreted much more

[2] Supplied by Charles E. Frosst and Company, Montreal, Canada, on allocation from the Atomic Energy Commission.

TABLE 1. EXCRETION OF C^{14} BY RATS GIVEN 50 μg. OF ESTRONE-16-C^{14}. VALUES ARE PERCENTAGES OF QUANTITY ADMINISTERED

Rats	Urine, per cent	Feces, per cent	Bile, per cent	Total, per cent
Normal-control-3-(IM)[1]	19.0	70.4		89.4
Normal-control-6-(IG)[2]	18.8	68.7		87.5
Duct-ligated-3-(IM)	93.4	0		93.4
Duct-ligated-3-(IG)	85.4	0		85.4
Duct-cannulated-3-(IM)	0.3	0	84.3	84.6
Duct-cannulated-3-(IG)	1.7	0	86.6	88.3

[1] IM—Intramuscular administration in dibutyl succinate.
[2] IG—Intragastric administration in absolute ethanol or a mixture of equal parts of water and 95% ethanol.

rapidly by the animals given the estrone-16-C^{14} intragastrically. The control and bile fistula animals excreted C^{14} for only 2 days while in the intramuscular series C^{14} was excreted for 4 or 5 days. The excretion of C^{14} by the duct-ligated animals was prolonged in both series but rats which had been given estrone intragastrically excreted very little after the second day.

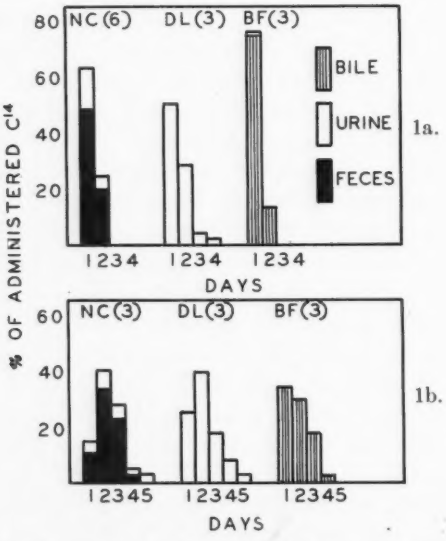

FIG. 1a. Average daily excretion of C^{14} after the intragastric administration of 50 μg. of estrone-16-C^{14} in 0.25 ml. of absolute ethanol or a mixture of equal parts of water and 95% ethanol. Fig. 1b. Average daily excretion of C^{14} after the intramuscular administration of 50 μg. of estrone-16-C^{14} in 0.20 ml. of dibutyl succinate. For both Fig. 1a and Fig. 1b, the height of the bar indicates total daily excretion. NC, rats with intact bile ducts; DL, animals with ligated bile ducts; BF, animals with cannulated bile ducts. The number of animals is given in parentheses.

In later experiments, the excretion after large amounts of estrone was studied; an animal with a bile fistula was given 380 μg. of radioestrone intragastrically. Eighty-two per cent of the C^{14} was excreted in the bile, 9% in the urine and a very small amount in the feces. A control animal was given 400 μg. of radioestrone intramuscularly; the expired air was assayed for radioactivity but none was detected. Twenty-one per cent of the radioactivity was found in the urine and 64% in the feces during 5 days.

Nature of Radioactive Products in Excreta. The 24-hour urines of the control animal which had been given 400 μg. of estrone-16-C^{14} intramuscularly were pooled, adjusted to pH 6 and extracted with ether to remove unconjugated estrogen. The urine was then extracted with butanol to remove conjugated estrogen metabolites, and the solvent distilled *in vacuo*. The residue was taken up in buffer at pH 6.4 and this aqueous solution used in the enzymatic experiments. It was incubated twice with a bacterial β-glucuronidase preparation (Katzman *et al.*, 1954) and twice with a phenolsulfatase preparation (Mylase P, Cohen and Bates, 1949) at 37° for 24-hour periods. The incubation mixture was extracted 5 times with ¼ volume of ether after each incubation.

Examination of the nature of the radiometabolites in bile was conducted in a manner similar to that for urine and included pooling the samples containing C^{14} from one animal and extracting with ether. Since control experiments had shown that the enzymes are effective in the presence of bile, the butanol extraction was omitted and the enzymes added directly to the bile, after which the mixture was continuously extracted with ether. The pooled bile samples from the second and third bile-fistula animals and from the animal receiving 380 μg. of estrone-16-C^{14} were treated in similar fashion, except that the chelating agent, Versene, was added to the incubation mixture to protect the β-glucuronidase from inactivation by heavy metals (Doyle, 1954). In addition, since cysteine was observed to prolong the activity of β-glucuronidase in bile, it was added to the last pooled samples mentioned above.

Examination of the data in Table 2 reveals that, in animals receiving 50 μg. of estrone-16-C^{14}, very little of the C^{14} in untreated bile was ether-extractable; about one-fourth of the total C^{14} became soluble in ether after hydrolysis with β-glucuronidase. Phenolsulfatase liberated an additional small amount from the water-soluble fraction. The total ether-extractable material including that liberated by enzymes amounted to only about 33% of the C^{14} excreted. The three fractions from urine were similar to those from bile. However, the proportion of ether-soluble metabolites in bile after hydrolysis was markedly increased after a larger amount of estrone-16-C^{14} was administered. Only 1.4% of the radioactivity was ether-extractable before incubation with enzymes. β-Glucuronidase released 63.8% of the total radiocarbon as ether-extractable material and

TABLE 2. ETHER EXTRACTION OF URINE AND BILE BEFORE AND AFTER HYDROLYSIS WITH ENZYME PREPARATIONS

Extract	Per cent of C^{14} ether-extractable				
	Urine[1]	Bile[2]			Bile[3]
Before enzymes	0.8	3.3	3.3	3.3	1.4
After β-glucuronidase	28.8	26.2	18.3	30.9	63.8
After phenolsulfatase	3.2	5.0	5.5	5.5	1.6
Total	32.8	34.5	27.1	39.7	66.8

[1] Rat given 400 μg. of estrone-16-C^{14} intramusculary.
[2] Rat given 50 μg. of estrone-16-C^{14}. The data in each column were obtained on the pooled biles of different rats; the first two columns of data are from animals in the intramuscular series, the third column from the intragastric series.
[3] Rat given 380 μg. of estrone-16-C^{14} intragastrically.

phenolsulfatase released another 1.6%. The increased amount of conjugate hydrolyzed by β-glucuronidase may have been due to the administration of the much larger amount of estrone.

To obtain more information about the metabolites, the extracts containing the free metabolites and those conjugates hydrolyzed by enzymes from the bile of each rat were combined. Each pooled sample was fractionated by the Friedgood procedure (Friedgood *et al.*, 1948) after having obtained satisfactory results on control mixtures of estrogens. After partition into neutral, strongly phenolic and weakly phenolic fractions, the weakly phenolic fraction was separated into non-ketonic and ketonic portions. Estrogenic activities of the fractions were determined by the Emmens intravaginal assay method. The data from these fractionations are listed in Table 3.

TABLE 3. RESULTS OF ASSAYS AFTER THE FRIEDGOOD FRACTIONATION OF ETHER EXTRACTS OF BILE

Expt. No.[1]	Neutral		Strongly phenolic		Weakly phenolic			
					Non-ketonic		Ketonic	
	% C^{14}	Estrogenic activity,[2] per cent	% C^{14}	Estrogenic activity, per cent	% C^{14}	Estrogenic activity, per cent	% C^{14}	Estrogenic activity, per cent
1	7.8	†	24.0	<20	1.7	50	27.9	33
2	7.3	†	21.8	<20	1.4	12	24.2	33
3	7.0	†	20.5	<20	2.5	50	46.0	33
4	5.4	2	22.4	10	1.1	50	33.4	33

[1] Animals in experiments 1, 2 and 3 given 50 μg. of estrone-16-C^{14} intramuscularly or intragastrically and animal in experiment 4 given 380 μg. of estrone-16-C^{14} intragastrically.
[2] The estrogenic activity of each fraction is expressed as the per cent of the possible estrogenic activity for that C^{14} content. For example, an aliquot of the weakly phenolic, non-ketonic fraction contained 6.2 d/m. This radioactivity, when compared with that of the original estrone, is equivalent to 0.001 μg. of estradiol-17β; on bioassay by the Emmens procedure, it produced a positive smear in 50% of the animals. The amount of estradiol-17β required for this response is 0.0005 μg. Therefore, the estrogenic activity of this fraction indicates that not more than 50% of the radioactivity in this "estradiol fraction" could be present as estradiol-17β.
† Samples not bioassayed.

The estrogenic activity of the ether-extractable material was rather weak and radioactive compounds having little or no estrogenic activity appeared in the estriol and estrone fractions. However, the latter fraction was found to contain the highest percentage of radioactivity and most of the estrogenic activity. The presence of C^{14} in the neutral fraction is also interesting and may be significant. The recovery of C^{14} in the phenolic and neutral fractions amounted to 64%. Another 18% was found in the aqueous residues. Bioassay of 2 bile samples after enzymatic hydrolysis and extraction with ether showed estrogenic activities of 7 and 8% expressed as estrone remaining in the aqueous phase.

The ether extracts from the bile of the animal given 380 μg. of estrone-16-C^{14} were also pooled and subjected to the Friedgood procedure. Additional extractions were made at almost every step to insure as complete extraction as possible. In addition the Girard separation was performed twice. The data are included in Table 3. It is evident that the pattern of fractionation of C^{14} and the bioassays are similar to those observed in the previous experiments. Bioassay of the neutral fraction showed it to have less than 2% of estrogenic activity calculated as estrone. The extracted aqueous fractions were found to contain 26% of the C^{14}.

The pooled ether extracts of the urine sample studied were partly fractionated. About 1% of the C^{14} was found in the neutral fraction and 23% in the strongly phenolic fraction. The estrogenic activity of the strongly phenolic fraction was 20% of that possible on the basis of the C^{14} content.

DISCUSSION

The data show that the patterns of excretion of radioactivity after the intramuscular or intragastric administration of 50 μg. of estrone-16-C^{14} are similar by both routes of administration. The administration of larger amounts of radioestrone did not alter this pattern much, although the urinary excretion of C^{14} in the bile-fistula animal increased with the greater dosage.

The exclusive urinary excretion of C^{14} in the duct-ligated animals shows that the kidney can and does take over the excretion of estrone metabolites when excretion by the liver is blocked. That the bile is the main excretory pathway of administered radioactivity in the rat is definitely established by the almost exclusive biliary excretion of C^{14}-labeled compounds in bile-fistula animals. The increase in urinary C^{14} in the one bile-fistula animal receiving 380 μg. of estrone-16-C^{14} might be explained on the basis that the large amount of estrone exceeded the hepatic capacity. Biliary excretion of C^{14} following the administration of C^{14}-labeled estrogens has been noted previously by Bocklage *et al.* (1953) in our laboratories using 17-methyl-C^{14}-estradiol, and by Hanahan *et al.* (1953) using C^{14}-diethylstilbestrol. Bocklage *et al.* found as much as 87% of administered radioactivity in bile, while Hanahan *et al.* found 53–87%. After the administration of

radioactive dibromoestrone (Br^{82}) to a dog, Twombly *et al.* (1948) found 69.8% of the radioactivity in the bile in 5 hours.

The absence of detectable C^{14} in the expired air of the animal given 400 μg. of estrone-16-C^{14} is good evidence that, in the rat, carbon atom 16 is not removed from the molecule.

The presence of 20% of C^{14} in the urine of control animals as compared with its almost complete absence in the urine of bile-fistula rats is indicative of resorption of C^{14}-labeled products which have been excreted in bile.

The results of extraction of bile show that very little of the radioactive biliary products is present in an ether extractable form, and in the rats given amounts of estrone-16-C^{14} in the physiological range only one-third is extractable with ether after hydrolysis with enzymes. Longwell and McKee (1942), working with dogs, recovered very little estrogenic activity (1.3–8%) in bile after the administration of estrone. Cantarow *et al.* (1942) reported that bioassay indicated almost complete recovery of estrogenic activity in the bile of dogs given estrone; but, in a continuation of this work (Pearlman *et al.*, 1948), the recovery of 4–22% of estrone and estradiol-17β, calculated on the basis of estrogenic activity, in the bile of dogs given estrone acetate was reported. Our experiments on rats show that after taking into consideration the estrogenic activities of the respective estrogens in the fractions of the total ether extracts only from 2.3–6.7% of the administered compound is accounted for by bioassay.

That very little, if any, of the estrone-16-C^{14} is converted to estriol is evident from the absence of any appreciable estrogenic activity in the strongly phenolic fraction. It is quite possible that some of the radioestrone is excreted unchanged in either the free or conjugated form or both, since the weakly phenolic, ketonic fraction contains a considerable part of the ether-extractable C^{14} products and most of the biological activity is found in this fraction. The presence of C^{14} in the neutral fraction is interesting and may be significant especially since one of these fractions exhibited very little estrogenic activity, less than 2% in terms of estrone.

SUMMARY

In the rat given estrone-16-C^{14}, the bile is the major excretory pathway of C^{14} excretion. If this route is blocked, the urine becomes the major excretory route.

Very little of these radioactive biliary products is in the free form and practically all of the ether-extractable material is obtained after hydrolysis with the enzymes studied. The administration of larger amounts of estrone-16-C^{14} results in an increased yield of ether-extractable products after enzymatic hydrolysis. The ether-extractable material has been shown to consist mainly of non-estrogenic compounds or compounds with very weak estrogenic activity.

REFERENCES

ALBERT, S., R. D. H. HEARD, C. P. LEBLOND AND J. SAFFRAN: *J. Biol. Chem.* **177:** 247. 1949.

BEER, C. T., K. DOBRINER, M. L. EIDINOFF AND T. F. GALLAGHER: *Fed. Proc.* **12:** 176. 1953.

BEER, C. T. AND T. F. GALLAGHER: *J. Biol. Chem.* **214:** 335. 1955.

BOCKLAGE, B. C., H. J. NICHOLAS, E. A. DOISY, JR., W. H. ELLIOTT, S. A. THAYER AND E. A. DOISY: *J. Biol. Chem.* **202:** 27. 1953.

BOLLMAN, J. L.: *J. Lab. and Clin. Med.* **33:** 1348. 1948.

BUDY, A. M.: *Fed. Proc.* **12:** 306. 1953.

CANTAROW, A., A. E. RAKOFF, K. E. PASCHKIS AND L. P. HANSEN: *Proc. Soc. Exper. Biol. and Med.* **49:** 707. 1942.

COHEN, H. AND R. W. BATES: *Endocrinolgoy* **44:** 317. 1949.

DAVIS, M. E., F. E. KELSEY, N. W. FUGO, J. E. LOUCKS, E. N. HORNER AND P. VOSKUIL: *Proc. Soc. Exper. Biol. and Med.* **74:** 501. 1950.

DINGEMANSE, E. AND E. LAQUEUR: *Am. J. Obst. and Gynec.* **33:** 1000. 1937.

DOISY, E. A., S. A. THAYER AND J. T. VAN BRUGGEN: *Fed. Proc.* **1:** 202. 1942.

DORFMAN, R. I.: *J. Biol. Chem.* **119:** xxv. 1937.

DOYLE, M. L.: *Fed. Proc.* **13:** 201. 1954.

EMMENS, C. W.: *J. Endocrinol.* **2:** 444. 1941.

FRIEDGOOD, H. B., J. B. GARST AND A. J. HAAGEN-SMIT: *J. Biol. Chem.* **174:** 523. 1948.

HANAHAN, D. J., E. G. DASKALAKIS, T. EDWARDS AND H. J. DAUBEN, JR.: *Endocrinology* **53:** 163. 1953.

HANAHAN, D. J. AND N. B. EVERETT: *J. Biol. Chem.* **185:** 919. 1950.

HEARD, R. D. H.: *Recent Progress in Hormone Research* **4:** 25. 1949; Acad. Press, N. Y.

KATZMAN, P. A., R. F. STRAW, H. J. BUEHLER AND E. A. DOISY: *Recent Progress in Hormone Research* **9:** 45. 1954; Acad. Press, N. Y.

LEBLOND, C. P.: *Ciba Foundation Colloquia on Endocrinology*, Philadelphia, Blakiston Co., vol. II, p. 153, 1952.

LEVIN, L.: *J. Biol. Chem.* **157:** 407. 1945.

LIEBERMAN, S. AND S. TEICH: *Pharmacol. Rev.* **5:** 285. 1953.

LONGWELL, B. B. AND F. S. MCKEE: *J. Biol. Chem.* **142:** 757. 1942.

PEARLMAN, W. H., M. R. J. PEARLMAN AND A. E. RAKOFF: *J. Biol. Chem.* **209:** 803. 1954.

PEARLMAN, W. H., A. E. RAKOFF, K. E. PASCHKIS, A. CANTAROW, AND A. A. WALKLING: *J. Biol. Chem.* **173:** 175. 1948.

SIEBKE, H. AND P. SCHUSCHANIA: *Zentr. Gynakol.* **54:** 1734. 1930.

SOLOMON, S. AND R. D. H. HEARD: *Rev. canad. biol.* **11:** 82. 1952.

STIMMEL, B. F.: *Fed. Proc.* **13:** 305. 1954.

THOMPSON, L. AND R. D. H. HEARD: *Rev. canad. biol.* **11:** 86. 1952.

TWOMBLY, G. H., L. MCCLINTOCK AND M. ENGELMAN: *Am. J. Obst. and Gynec.* **56:** 260. 1948.

THE COMPARATIVE ACTIVITIES OF $\Delta^{1,4}$ PREGNADIENE-21-OL-3,20-DIONE ACETATE AND DESOXYCORTICOSTERONE ACETATE IN THE RAT[1]

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UNSATURATION of the 1:2 linkage of the cortical steroids has been reported to modify their pharmacologic properties. Thus the compounds $\Delta^{1,4}$ Pregnadiene-17α,21-diol-3,11-20-trione and $\Delta^{1,4}$-Pregnadiene-11β,17α,21-triol-3,20-dione, respectively analogues of cortisone and hydrocortisone (Herzog *et al.*, 1955), have greater adrenocortical, antirheumatic and anti-inflammatory activities (Bunin *et al.*, 1955) though their side reactions are not correspondingly increased. This observation indicates that partial dissociation of pharmacological activities may result from minor changes in the structure of the steroid nucleus. The present study is an investigation of the effects of a like nuclear change in desoxycorticosterone (DCA). For this, we have compared the activities of $\Delta^{1,4}$-Pregnadiene-21-ol-3,20-dione Acetate (PODA)[3] with those of DCA (Δ^{4}-Pregnene-21-ol-3,20-dione) in adrenalectomized rats and in rats under the conditions used for the production of "DCA-hypertensive disease" (Selye *et al.*, 1943; Masson *et al.*, 1950).

A. EFFECT IN ADRENALECTOMIZED RATS

Methods

Fifty-eight male rats, weighting 70–90 gm. were divided into 7 groups as shown in Table 1. Their diet was Purina Fox Chow and water *ad lib.* Adrenalectomy was carried out under surgically clean conditions using ether anesthesia. Subcutaneous injections of steroid in peanut oil, or of peanut oil alone were given twice daily in 0.1 ml. doses. The rats were weighed daily and when half of the animals in the control group had died, i.e., on the sixth day, all the remaining animals were killed by withdrawing blood from their carotid arteries under ether anesthesia. The blood was used to determine the hematocrit ratio, and urea nitrogen (Archibald, 1945) and plasma chloride concentrations (Van Slyke and Hiller 1947).

Results

These are summarized in Table 1. Sixteen rats received peanut oil alone and these failed to grow. They developed a high blood urea nitrogen

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TABLE 1. COMPARATIVE EFFECTS OF PODA AND DCA IN ADRENALECTOMIZED RATS[1]

Daily treatment	Number of rats	Weight change by the 6th day of the experiment, gm.	Blood urea, mg./100 ml.	Plasma chloride, mM/L.	Hematocrit, per cent
Peanut oil	16[2]	− 2±1.8	38±4.7	102±2.9	44±1.1
DCA					
150 mg.	8	+32±2.42	13± .7	113±1.3	33±1.19
75 mg.	6	+29±3.34	12±1.11	105±3.13	33±1
PODA					
1 mg.	8	+39±1.19	12±3.16	106± .8	34±2
500 mg.	8	+34±1.41	14± .57	106±1.7	32±1.7
150 mg.	8	+31±1.89	12± .7	106±3.12	33± .77
75 mg.	8	+22±2.58	17± .53	107± .77	36± .77

[1] In this table as in table 2, the values are given with the standard errors.
[2] 8 of these rats died on or before the 6th day of the experiment—for these, the weight change by the time of death is used.

(mean, 38 mg./100 ml.) and a high hematocrit ratio (mean, 44%); the plasma chloride remained relatively low (mean, 102 mM/L.).

Fourteen rats received DCA in peanut oil: eight were given daily 150 μg. and the other six 75 μg. The rats on the higher dose gained weight and showed some hyperchloremia (mean, 113 mM/L.); the hematocrit ratio was low (mean, 33%) but the blood urea nitrogen was normal. The rats on the lower dose of DCA gained less weight and showed no hyperchloremia; otherwise they behaved similarly to the animals in the previous group.

Twenty-eight rats received PODA in peanut oil. The daily doses were as follows: in eight 1 mg.; in eight 500 μg.; in six 75 μg. All the rats gained weight and the gains were greater when the doses were larger. Except in the rats on the lowest dose of PODA, the blood urea nitrogen, plasma chloride and hematocrit levels were not significantly different from those found in the rats receiving 75 μg. per day of DCA. The rats on 75 μg. of PODA, however, gained less weight than the other animals receiving steroids, showed slight urea retention and a relatively higher hematocrit ratio (mean, 36%).

Thus it would appear that doses of PODA down to 150 μg. per day are effective, at least for short periods, in maintaining the health of adrenalectomized rats. In this respect the compound seems to be almost as efficient as DCA; the data also suggest that its chloride-retaining activity may be somewhat less than that of DCA.

B. HYPERTENSION-PRODUCING EFFECT

Methods

Unilateral nephrectomy under ether anesthesia was carried out in 34 male rats weighing about 100 gm. The animals, divided into 4 groups as shown in Table 2, were treated

as follows: Group I, untreated; Group II, DCA; Group III, PODA; Group IV, DCA combined with PODA. The steroids were administered in the form of 35 mg. pellets, two of each compound used being implanted subcutaneously at the time of the nephrectomy. Postoperatively, the animals were housed in metabolism cages on a diet of Fox Chow with 1% saline solution to drink. Their urine output was measured daily and the weight and blood pressure (del Greco *et al.*, 1953) recorded at approximately 5-day intervals. After four weeks, when hypertension was well established in the test animals, all surviving rats were killed and the same blood estimations performed as in the adrenalectomy experiment. Only 8 animals failed to survive the full time, 2 from group II and 3 each from groups III and IV; these died during the fourth week. *Post mortem* careful search was made for cardiovascular lesions. The kidney, heart, spleen and adrenals were fixed in Zenker's fluid for subsequent dissection, weighing and histological examination. Paraffin sections of these organs and also of samples of mesentery and intestine were stained with hematoxylin and eosin, and by the periodic acid-Schiff method.

Results

These are summarized in Table 2. All three groups of rats with subcutaneous steroid implants gained much less weight than the control group and developed severe hypertension, polyuria, a reduction of the hematocrit ratio and slight hyperchloremia. The blood urea nitrogen in the control group was at the upper limit of normal; the other groups showed some azotemia which was most marked in the rats receiving the combined treatment. Groups II, III and IV also showed increases in the weights of the kidney and heart (estimated as a proportion of the body weight of the

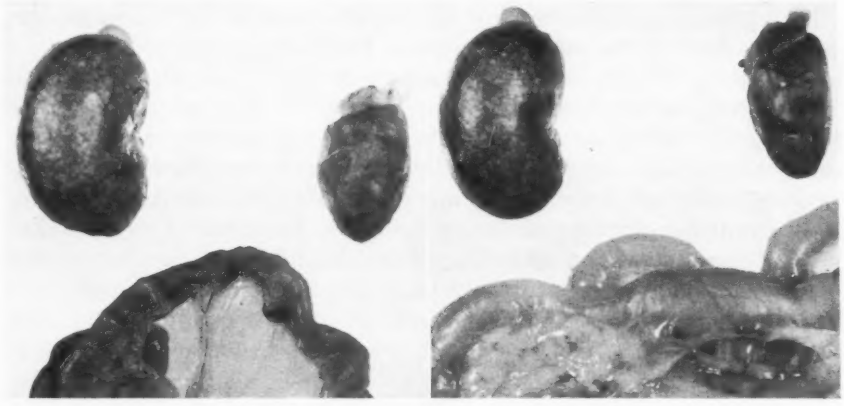
TABLE 2. COMPARATIVE EFFECTS OF PODA AND DCA IN UNINEPHRECTOMIZED RATS ON 1 PER CENT SALINE

Group Treatment		I No extra treatment	II DCA	III PODA	IV DCA +PODA
Number of rats		7	9	9	9
Body weight	Initial	100 ±3.5	100 ± 2.8	100 ± 3.2	100 ± 2.9
	Final	247 ±5.1	167 ± 4.3	176 ± 7.5	141 ± 5.2
Blood pressure	5th day	139 ±7.9*	163 ± 8.3	166 ± 6.2	170 ± 7.2
	15th day	135 ±5.9*	191 ±12.3	188 ±10.8	188 ± 9.0
	30th day	128 ±1.9*	215 ±15	207 ±11.7	215 ±10.8
Average daily urine output during	1st week	31 ±2.96	48 ± 2.7	48 ± 8.4	57 ± 7.7
	2nd week	38 ±4.29	96 ±12.5	117 ± 7.9	105 ±11.1
	4th week	44 ±2.21	115 ±15.1	122 ±11.1	99 ±13.9
Hematocrit per cent		44 ±0.92	33 ± 3.2	34 ± 2.7	29 ± 2.4
Plasma chlorides, mM/l.		104 ±1.89	110 ± 1.9	109 ± 1.8	112 ± 1.7
Blood urea nitrogen, mg. per cent		22 ±1	25 ± 4	31 ± 5.3	39 ± 3.5
Weight of organs mg. per 100 gm. body weight	Kidney	670 ±12	1280 ±120	1210 ±125	1290 ±130
	Heart	290 ±6	530 ±40	580 ±22	540 ±21

* One rat was eliminated because of consistent spontaneous hypertension (blood pressure 160–170 mm.). At necropsy the only demonstrable lesion that might have caused this was a moderate degree of hydronephrosis in the remaining kidney.

animals at the time of death): these values were approximately double those found in the control group. The general condition of the rats of group IV deteriorated more rapidly than that of the other groups and during the last week of the experiment their urine output dropped; this is attributed to their cachectic state rather than to cessation of steroid diuresis.

Pathologically, gross evidence of severe hypertensive disease was found in nearly all the animals from the test groups. Only three rats failed to show macroscopic lesions (two from group II and one from group III), and these had microscopic renal changes. Lesions were not detected in the control group. In the three groups treated with steroids, there was little



A. *B.*

FIG. 1. Hypertensive vascular lesions in the kidney, heart and mesentery of uninephrectomized rats on 1 per cent saline. A. treated with DCA B. treated with PODA. Note the granularity of the surface of the kidney, the white patches in the myocardium and the small nodules at the periphery of the mesentery. In (B) the heart shows hemorrhages and the mesenteric lesions are more severe (×1.6).

difference in the intensity of the lesions. They were somewhat more severe in rats given PODA or PODA with DCA than in those receiving DCA alone. Since PODA was somewhat less active than DCA when given by injection to the adrenalectomized animals this difference is the reverse of what we had anticipated.

The lesions were found mainly in the kidneys, heart, mesenteric vessels and gastro-intestinal tract (Fig. 1); they were of the same type as those previously seen in "DCA hypertensive disease" in the rat (Masson *et al.*, 1950). *The kidney* was granular and often showed a few subcapsular petechiae with occasional flame-shaped hemorrhages on the cut surface. Histologically, there was a notable eosinophilic exudate around and between the loops of the glomerular tuft and similarly-staining albuminous material was present in abundance as casts in the renal tubules. Many of

the arterioles showed degrees of fibrinoid damage varying from minimal tinctorial changes to gross occlusive necrosis. *The heart* showed some surface nodularity associated with opaque white patches, particularly near the apex of the right ventricle. Histologically, the affected area showed arteriolar lesions varying from minor perivascular infiltration by mononuclear cells to gross fibrinoid necrosis and cellular infiltration of the adjacent myocardium. *The mesenteric arteries* often showed a conspicuous beading due to focal mural necroses and intense perivascular leucocytic infiltration.

COMMENT

The present experiments show that modification of the steroid nucleus of DCA by 1:2 unsaturation to form PODA, produces no more than minor changes in pharmacologic properties. PODA, administered in oil by injection, showed slightly less capacity for the maintenance of health in adrenalectomized rats; hyperchloremia did not occur even when large doses were used. When pellets of the compound were implanted subcutaneously in uninephrectomized rats, given 1% saline to drink, the capacity to induce hypertensive vascular lesions appeared to be somewhat greater than that shown by DCA pellets. The differences in activity may merely reflect differences in the rate of absorption of the steroids from the oily solutions and from the pellets, though this aspect has not been investigated further.

SUMMARY

The comparative effects of $\Delta^{1,4}$ Pregnadiene-21-ol-3,20-dione Acetate and Desoxycorticosterone Acetate have been studied in adrenalectomized rats and in rats made susceptible to hypertensive disease by unilateral nephrectomy and saline administration. Approximately the same pharmacological effects were observed with both compounds: $\Delta^{1,4}$ Pregnadiene-21-ol-3,20 dione maintained health in the adrenalectomized rats at a slightly higher dose level than the desoxycorticosterone acetate; like desoxycorticosterone acetate it also induced severe hypertensive vascular disease in the unilaterally nephrectomized rats.

REFERENCES

ARCHIBALD, R. M.: *J. Biol. Chem.* **157:** 507. 1945.

BUNIN, J. J., M. M. PECHET AND A. J. BOLLET: *J.A.M.A.* **157:** 311. 1955.

HERZOG, H. L., A. NOBILE, S. TOLKSDORF, W. CHARNEY, E. B. HERSHBERG AND P. L. PERLMAN: *Science* **121:** 176. 1955.

MASSON, G. M. C., J. B. HAZARD, A. C. CORCORAN AND I. H. PAGE: *Arch. Path.* **49:** 641. 1950.

SELYE, H., C. E. HALL AND E. M. ROWLEY: *Canad. M. A. J.* **49:** 88. 1943.

VAN SLYKE, D. D. AND A. HILLER: *J. Biol. Chem.* **167:** 107. 1947.

RESPONSES OF OVARIES OF IMMATURE CHICKENS TO AVIAN AND MAMMALIAN GONADOTROPHINS[1]

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THE bulk of studies on the response of avian gonads to gonadotrophic hormones has been done using hormones of mammalian origin. This work established that gonads of normal adult chickens of both sexes are capable of being stimulated by mammalian gonadotrophins. However, in the domestic chicken, between the ages of one and about 150 days, the gametogenic component of the ovary is almost completely refractory to gonadotrophins of mammalian origin. Thus, injection of crude or purified gonadotrophic substances from sheep, pigs and mares (including pregnant mare's serum) does not lead to any measurable enlargement of ovarian follicles of prepuberal female chickens. Two alternative explanations for the failure of the prepuberal avian cortex to respond to mammalian hormones can be offered: 1. a physiological or morphological unreadiness of the cortex of the avian ovary, and 2. an endocrine incompleteness of the mammalian hormones used.

The second possibility was investigated in this study by comparing the effects of avian and mammalian gonadotrophic hormones on the prepuberal chicken ovary. The results of this work are the subject of this paper.

Nalbandov *et al.* (1951) found that hypophysectomized male chickens are capable of responding to mammalian gonadotrophins for a very limited time (10–14 days), whereas sustained testicular growth and androgen secretion could be obtained by the injection of chicken pituitaries alone or in combination with mammalian hormones. This led to the interpretation that the avian gonadotrophic complex either consists of three gonadotrophic hormones, or that the avian luteinizing hormone (LH) may possess properties not shared by mammalian LH. Because this work demonstrated

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[2] Data taken from a thesis presented to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philsosophy. Present address: Indian Statistical Institute, 203 Barrackpore Trunk Road, Calcutta-35, India.

a significant qualitative difference in the gonadotrophic complex of mammals and birds by using hypophysectomized males as experimental animals, it became of interest to see if this difference applied to female chickens as well.

The inability of the cortical portion of prepuberal chicken ovaries to respond to mammalian gonadotrophic hormones has been noted (Domm, 1933; Asmundsen and Wolfe, 1935; Benoit, 1937; Nalbandov and Card, 1946 and Taber, 1948). Some of the workers noted an increase in the number of follicles as a result of the injection of mammalian gonadotrophic hormones but in all cases the stimulated follicles were of microscopic size, few if any of them reaching the size of 1 mm. Riley and Fraps (1942) assayed pituitary glands from chickens in various reproductive stages and concluded that glands from hens with regressed or quiescent ovaries produced a greater gonad stimulating effect in immature mice than did those from hens in full reproductive conditions.

MATERIALS AND METHODS

Female chickens (*Gallus domesticus*) of the White Leghorn and Rhode Island Red breeds were used. They ranged in age from 45 to 131 days at the start of the different experiments. The younger ages (45, 70 and 93 days) were selected because such birds have completely infantile ovaries which contain only minute follicles. The older ages (110 and 130 days) are somewhat closer to sexual maturity which, in the breeds used, occurs at about 150 to 180 days of age. Their follicles are still microscopic in size although the ovaries are much larger at this age. Thus, we were able to compare effectiveness of hormone treatment on ovaries of two distinctly different physiological age groups and to determine whether "physiological maturity" is a prerequisite to a response to gonad stimulation.

Because follicular growth in chickens involves a major job of mobilization, transportation and deposition of large quantities of the lipo-protein precursors of yolk, it was thought necessary to extend the treatments over a sufficient length of time to insure maximum effectiveness. In the normal mature laying hen the task of increasing yolk size from about 100 mg. to 16 gm. requires about nine days. For this reason the duration of treatment selected for immature birds used in this study was 10, 15, 20 and 35 days depending on the objective of the experiment.

In each experiment birds of the same brood, age and breed were allotted at random to treatment groups and to cages. To minimize possible inequalities of light intensity, birds were reassigned every fifth day randomly by treatment groups to different cages at the time of weighing. Comb growth was recorded as volume (length $\times$ height $\times$ thickness). Some of the animals were hypophysectomized according to the method of Nalbandov and Card (1943).

When, at the termination of the injection periods the birds were killed, the ovaries and oviducts were collected and cleaned. After the follicles were measured and counted the ovaries were preserved in Bouin's fluid for 24 hours and weighed after having been cleaned.

Chicken pituitary glands were collected at a local killing plant and came from birds of both sexes varying in age from $2\frac{1}{2}$ to $3\frac{1}{2}$ months. These glands were pooled. In one experiment pituitary glands were obtained from non-laying old hens and from males and females 13 weeks of age. The glands in these groups were kept separate by age of donor.

It was most expedient to freeze the heads, later remove the glands from the thawing head and to refreeze the glands in a double-jacketted bottle kept in a mixture of dry ice and acetone. The glands were kept frozen until needed when they were crushed between glass plates and air-dried in a desiccator. Because there was some loss of potency, the dosages used were rather high and ranged from 6 to 16 mg. per bird per day.

The mammalian gonadotrophic hormone used was a partially purified preparation from sheep pituitaries containing both FSH and LH and was injected at the rate of 1.0 to 1.5 Armour units per bird per day. These dosages were selected after preliminary experiments had shown that they produced maximal or near maximal responses on the testes of immature male chickens and the ovaries of mature laying hens. All hormones were injected in a volume of 0.25 ml. of distilled water per injection.

Histological Technique

Ovaries were embedded in paraffin and sectioned sagittaly at 10 μ. The first 40 sections were discarded while the next four were mounted; the next 40 sections were again discarded and the next four mounted. All slides were stained in Mallory's triple stain. The sections were projected on paper (magnified 40×) and outline drawings of the ovarian cortex and medulla were made, their areas being determined with a planimeter for appropriate statistical analyses.

RESULTS AND DISCUSSION

Both the avian and the mammalian gonadotrophic complexes contain follicle stimulating hormone (FSH) and luteinizing hormone (LH), and both of these are known to have growth promoting effects on ovaries of chickens. The avian ovary consists of a medullary portion which can be stimulated to grow, presumably largely by LH although FSH also may exert stimulatory effects. It is primarily this effect on the medullary portion which is measured when ovaries of immature chickens, stimulated with mammalian gonadotrophic hormones, are weighed. The main concern of this study was not to cause an indiscriminate increase in ovarian weight, but to induce proliferation of the cortical portion of the ovary which gives rise to follicles. Thus, the main criterion to be considered here is not the ovarian weight but the number and size of ovarian follicles in which yolk deposition has been induced by the injection of either mammalian gonadotrophic hormones or of chicken pituitary glands.

The basic difference between the effects of mammalian and avian gonadotrophic complexes is shown when the two types of hormones are injected into very young, 45-day old, hypophysectomized females (Table 1). It is seen that only chicken pituitary powder was capable of inducing follicular growth. In normal birds somewhat closer to sexual maturity (110 days), the greatest effect on follicle number and follicle size was again produced by chicken pituitary powder. In still older but hypophysectomized females (Table 1), mammalian hormone was almost totally ineffective while chicken pituitary powder was capable of restoring comb size, ovarian weight and follicle size to within the range of the normal controls while it doubled the number of follicles stimulated.

These facts are interpreted to mean that the ovaries of immature female

TABLE 1. EFFECTS OF MAMMALIAN GONADOTROPHIN AND CHICKEN PITUITARY POWDER ON COMB SIZE, OVARY WEIGHT, AND FOLLICULAR DEVELOPMENT OF IMMATURE FEMALE CHICKENS

Age at first injection (days)	Duration of treatment (days)	Mammalian gonadotrophin (MG) Chicken pit. pwd (CP) daily dose	Comb size: % of initial size	Ovary weight and standard error (mg.)	Proportion of birds showing follicular growth	Total No. of follicles produced	Mean size of follicles present (cm.)
45	20	Intact Control	237	304 ± 25	0/5	0	—
		Hypox + MG (1.0 unit)	292	314 ± 27	0/5[3]	0	—
		Hypox + CP (14 mg.)	462	393 ± 30	3/5	6	0.16
93	10	Intact Control	141[1]	632 ± 2.0[1]	0/5	0	—
		Intact + CP (4 mg.)	392	886 ± 3	0/5	0	—
		Intact + CP (8 mg.)	473	737 + 77	3/5	4	0.28
		Intact + CP (16 mg.)	316	490 ± 27	5/5	10	0.28
110	35	Intact Control	521	1,100 ± 77	4/5	8	0.22
		Intact + MG (1.5 units)	633	950 ± 90	2/5[4]	5	0.10
		Intact + CP (8 mg.)	567	15,540 ± 818	5/5	46	0.92
131	20	Intact Control	279[2]	9,815 ± 5,977	3/5	22	1.06
		Hypox Control	31	413 ± 6[2]	0/5[4]	0	—
		Hypox + MG (1.5 units)	55	606 ± 6	0/5	0	—
		Hypox + CP (8 mg.)	117	7,748 ± 3,867	5/5	46	0.78
70	15	Intact Control	123[2]	316 ± 10[2]	0/5[3]	0	—
		Intact + CP[1] (10 mg.)	176	403 ± 36	0/5	0	—
		Intact + CP[2] (10 mg.)	242	505 ± 78	2/5	8	0.14
		Intact + CP[3] (10 mg.)	245	775 ± 86	2/5	6	0.16

Pituitaries from:
CP[1] = Non-laying hens.
CP[2] = 13 wk. old females.
CP[3] = 13 wk. old males.
[1] $P < .05$ on original size or weight.
[2] $P < .01$ on original size or weight.
[3] $P < .05$ on total number of birds showing follicles.
[4] $P < .01$ on total number of birds showing follicles.

chickens are capable of responding to gonadotrophic stimulation. The fact that they do so only when chicken pituitary powder is administered shows that there is a qualitative difference between the mammalian and avian gonadotrophic complexes. These findings are in accord with the earlier work done in this laboratory on hypophysectomized male chickens which also showed a distinct qualitative difference in their response to gonadotrophic complexes from the two sources of hormone under analysis. Neither this study nor the previous one on male chickens answer the question whether the difference is due to a third gonadotrophic hormone (other than FSH and LH) present in avian glands or whether it is due to a qualitative difference between avian and mammalian LH.

A further illustration of the qualitative difference under discussion is provided by a comparison of the relative effects produced by the two types of hormone on the component parts of the ovary. It is seen (Table 2) that mammalian hormones did cause an increase in ovarian cortical area over that of untreated hypophysectomized controls but had almost no effect on the medullary area. Chicken pituitary powder, on the other hand, caused a very significant increase in cortical area and, what is more significant, was able to restore the medullary area to that found in the ovaries of intact controls.

It should be noted that there was a further qualitative difference within chicken pituitary powder obtained from different sexes and ages. Thus, pituitary glands from non-laying hens did not contain the factor necessary

for follicular growth even though they were capable of acting on the ovarian cortex, thus causing both comb growth and an increase in ovarian weight. This does not agree with the conclusions of Riley and Fraps (1942) that pituitary glands from non-laying hens are more potent in gonadotrophin than those of laying birds, although these findings may not be contradictory in view of the fact that Riley and Fraps used immature female mice as their assay animals, judging gonadotrophic potency by the increment in ovarian and uterine weight, while we used follicular growth in the chicken as the index of gonadotrophic potency. In contrast, the

TABLE 2. EFFECT OF MAMMALIAN GONADOTROPHIN (MG) AND CHICKEN PITUITARY POWDER (CP) ON THE OVARIAN CORTEX AND MEDULLA OF PREPUBERAL CHICKENS

Treatment and number	Average cortical		Average medullary		Average ovarian weight (mg.)
	Area ($cm.^2$)	Percent	Area ($cm.^2$)	Percent	
5 Normal controls	17.76	100	5.19	100	9,815
5 Hypophysectomized controls	11.44	64	2.81	54	413
5 Hypox+MG	14.74	83	2.88	55	605
5 Hypox+CP	24.08	136	5.60	108	7,778
Significance level P	0.5		0.5		

hypophyses of male and female chickens killed at an age of 13 weeks, contain the factor required for follicular growth which is missing in the pituitary glands of mammals and non-laying hens.

Apparently, in immature female chickens, endogenous gonadotrophin is not secreted or released into the blood stream in sufficiently high concentrations to permit follicular maturation. The factor (third gonadotrophic hormone?) which is essential for follicular development in chickens is not secreted or released until near the time of puberty thus explaining the inability of immature females to respond to mammalian gonadotrophins. In mature, laying hens mammalian gonadotrophins can induce follicular hypertrophy and hyperplasia because the animal's own pituitary secretes the whole avian gonadotrophic complex which also contains the factor essential for follicular maturation.

SUMMARY

Transformation of follicles, from microscopic to macroscopic size including yolk formation, could be induced by the administration of chicken pituitaries to intact or hypophysectomized prepuberal female chickens. In contrast, follicular development could not be caused by mammalian gonadotrophin in either intact or hypophysectomized prepuberal or mature females. Exogenous mammalian gonadotrophins did induce follicular development in females approaching sexual maturity.

Gonadotrophins of either source could act on the medullary portion of the ovary, causing an increase in ovarian weight and in comb growth.

The refractory condition of the cortical portion of the immature chicken ovary is not due to morphological or physiological unreadiness to respond to gonadotrophic hormones but is probably due to a qualitative inadequacy of the mammalian pituitary gonadotrophic complex.

The factor which causes follicular development in prepuberal female chickens is present in immature male and female pituitaries but is not present in the same concentration in pituitary glands of non-lying hens.

These studies support the idea that there is a qualitative difference between the avian and mammalian gonadotrophic complexes. Whether the avain hypophysis secretes a third gonadotrophic factor, or whether avian FSH or LH have properties not present in their mammalian counterparts, remains to be found.

REFERENCES

ASMUNDSON, V. S. AND M. I. WOLFE. *Proc. Soc. Exper. Biol. Med.* **32**: 1107. 1935.
BENOIT, J. *Bull. Biol. France Belg.* **71**: 394. 1937.
DOMM, L. V. *Proc. Soc. Exp. Biol. Med.* **31**: 356. 1933.
NALBANDOV, A. V. AND L. E. CARD. *Jour. Exp. Zool.* **94**: 387. 1943.
NALBANDOV, A. V. AND L. E. CARD. *Endocrinology* **38**: 71. 1946.
NALBANDOV, A. V., R. K. MEYER AND W. H. MCSHAN. *Anat. Rec.* **110**: 475. 1951.
RILEY, G. M. AND R. M. FRAPS. *Endocrinology* **30**: 537. 1942.
TABER, E. *Jour. Exp. Zool.* **107**: 65. 1948.

A QUANTITATIVE STUDY OF ADRENAL INFLUENCES UPON THE CELLULAR ELEMENTS OF BONE MARROW[1]

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ALTHOUGH the role of the adrenal in regulating peripheral blood cell numbers is still being explored (reviews by Gordon and Charipper, 1947; Daughaday, Williams and Daland, 1948; Aschkenasy, 1952; Grant and Root, 1952; Dougherty and Dougherty, 1953; Gordon 1954; Gordon, 1955; Swingle and Kleinberg, 1955), only scant attention has been paid to its influence upon the blood forming organs. Some investigators have reported stimulation whereas others have noted hypoplasia of the bone marrow following activation of the pituitary-adrenal axis. Thus, adrenalectomy in rats (Gordon, Piliero and Landau, 1951) and mice (Stein and Martin, 1951) results in a decreased erythroid-myeloid cell ratio in marrow. Adrenal cortical extract and cortisone prevent this change in the adrenalectomized rat (Gordon, Piliero and Landau, 1951). Moreover, erythropoietic stimulation has been observed following administration of adrenal cortical extract to intact rats (Hayes and Baker, 1952), after treatment of intact guinea pigs with ACTH (Hudson, Herdan and Yoffey, 1952), cortisone, hydroxycorticosterone (Yoffey, Ancill, Holt, Owensmith and Herdan, 1954) and desoxycorticosterone (Cicconardi and Lorusso, 1946), and in adrenalectomized rats receiving corticosterone (Fruhman and Gordon, 1955). In contrast, depressive effects upon hemopoiesis have been noted in intact rats injected with cortisone (Cavallero and Braccini, 1951; Thiersch, Conroy, Stevens and Finch, 1952) or ACTH (Baker and Ingle, 1948; Robbins, Cooper and Alt, 1955). Betz (1953) found that cortisone inhibited regeneration of hemopoietic tissues in rats following irradiation.

The present experiments describe the effects of adrenalectomy and replacement treatment with some adrenal steroids upon the bone marrow of the rat. It is felt that the application of quantitative techniques to this

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problem will help to clarify some of the existing discrepancies in the literature. Since Gordon, Piliero and Landau (1951) indicated that whole adrenal cortical extract, a complex steroid mixture, provided the most complete treatment for maintaining the structure of bone marrow in the adrenalectomized rat, mixtures of 11-oxy and 11-desoxy adrenal steroids were used to determine whether synergistic actions exist between these classes of substances.

METHODS

Intact male rats of a modified Long-Evans strain and weighing approximately 200 gms. were injected daily with the free alcohols of hydroxycorticosterone, corticosterone and desoxycorticosterone. Adrenalectomy was performed on the third day. Similar subcutaneous injections were continued on this day and for the subsequent four days. Rats were killed on the eighth day. A group of animals was adrenalectomized and given daily injections of 1 ml. saline, the vehicle for the steroids. The various groups and treatments are listed in Table 1.

A quantitative method has been employed for the examination of the bone marrow of the rat (Fruhman and Gordon, 1955). Rats were anesthetized with ether, exsanguinated by heart puncture and decapitated. The femurs were removed, cleaned of surrounding tissues and the bone mass determined before and after demedullation. The difference between these weights yielded the weight of the total femoral marrow. For this procedure the femur was split along its main axis and, after taking a portion of the bone marrow, the remainder of the marrow was removed by swabbing the bone cavity clean with squares of lens paper. A sample of the marrow was drawn into a calibrated 20 cu. mm. pipette. This marrow mass was determined by weighing the pipette before and after filling. The marrow was expelled into a tube containing 2 ml. of homologous serum and uniformly suspended by drawing the marrow up and down in a rubber-nippled pipette. Samples were then taken for hemacytometer counts and smears. One sample was diluted with Tyrode's solution and the second with Randolph's staining fluid. The first count provided an estimate of all cell types, the second of nucleated cells and eosinophils. The difference between the two counts represented the numbers of non-nucleated red blood cells in the sample. Moreover, using myelogram percentages obtained from stained smears in conjunction with the above procedures, the numbers of

TABLE 1. PLAN OF EXPERIMENTAL TREATMENTS

Group	No. rats	Daily treatment
1*	9	none
2	6	1 ml. 0.85% NaCl
3	8	0.5 mg. hydroxycorticosterone[1]
4	7	2.0 mg. hydroxycorticosterone
5	8	4.0 mg. hydroxycorticosterone
6	8	6.0 mg. hydroxycorticosterone
7	6	2.0 mg. corticosterone[2]
8	6	1.5 mg. corticosterone + 0.5 mg. DOC[3]
9	3	0.75 mg. corticosterone + 0.75 mg. hydroxycorticosterone + 0.5 mg. DOC
10	3	1.5 mg. corticosterone + 1.5 mg. hydroxycorticosterone + 1.0 mg. DOC
11	6	3.0 mg. hydroxycorticosterone + 1.0 mg. DOC

* Rats of Group 1 were untreated: Groups 2 to 11 were adrenalectomized.

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other cell types were computed. Nucleated erythroid cells were identified from bone marrow smears treated with Ralph's hemoglobin stain and counterstained with Harris' hematoxylin. Wright-stained smears were also examined. Slides were studied without knowing the treatment the rat had received. A minimum of 500 cells was counted for each slide.

Tibiae were fixed in Helly's fluid, decalcified by a formic acid-ion exchange resin (Win-3000), sectioned at 10μ in paraffin and stained with hematoxylin and eosin.

RESULTS

Following adrenalectomy no significant changes were seen in the concentrations of nucleated cells (Table 2). With the lower doses of the adrenal steroids used there were no alterations in the numbers of nucleated cells, but with the higher doses (Groups 4, 5, 6, 7 and 11) sharp reductions in cellular numbers occurred.

Further examination of Table 2 reveals that the various cell types of marrow were affected quite differently. Five days after adrenalectomy the concentration of nucleated red blood cells in the marrow was reduced to

TABLE 2. EFFECTS OF ADRENALECTOMY AND REPLACEMENT TREATMENT ON BONE MARROW CELL NUMBERS

(Means ± standard errors)

Group[a]	Millions Nucleated Cells/mm.^{3}	Millions Non-nucleated RBC/mm.^{3}	Thousands Nucleated RBC/mm.^{3}	Thousands Neutrophils /mm.^{3}	Thousands Eosinophils/mm.^{3}	Thousands Lymphocytes/mm.^{3}	Thousands Unclassified Cells/mm.^{3}
1	2.52±0.06	1.12±0.11	521± 59	1160± 58	127±14	445±47	277±39
2	2.48±0.03	0.78±0.10	249± 46†	963± 49*	197±20*	576±75	495±48†
3	2.47±0.11	0.60±0.08	592± 76†	1057± 80	140± 9*	392±58	289±92
4	2.15±0.11*	1.84±0.28†	772± 62†	965± 76	68± 6†	123±39†	272±53*
5	1.86±0.08†	2.17±0.39†	546± 48†	933± 90	37± 3†	138±27†	206±29†
6	1.94±0.09†	3.01±0.53†	792± 84†	839±102	37± 3†	160±23†	112±30†
7	2.33±0.05*	1.34±0.17*	655± 77†	1121± 92	26±10†	187±29†	341±43*
8	2.40±0.18	1.11±0.08*	819±218*	1044± 89	101±12†	135±48†	301±38*
9	2.17±0.15	1.70±0.44	617± 75†	929±193	93± 5†	202±73*	329±40*
10	1.98±0.25	2.20±0.40†	719±162†	837± 52	43± 3†	131± 8†	240±51†
11	2.13±0.08*	2.48±0.31†	711±106†	897± 73	41± 8†	157±45†	324±37

[a] Group 1 served as the control for Group 2. Group 2 is the control for Groups 3–11.
* $p = <0.05$ but >0.01.
† $p = <0.01$.

about 50% of normal. Replacement treatment with the steroids, given alone or in combination, resulted in marked increases in the numbers of these elements as well as in the numbers of non-nucleated red blood cells.

The concentrations of neutrophils displayed a tendency to decrease following adrenalectomy. With replacement treatment no statistically significant changes were noted although a trend toward decreased numbers of neutrophilic cells was evident in animals receiving the higher doses of steroid (Groups 6, 10 and 11).

At 5 days after adrenalectomy increased numbers of eosinophils were present in the marrow. In addition, every type of adrenal steroid treatment given (Groups 3–11) reduced significantly the eosinophil levels. In most cases the numbers of eosinophils were decreased to values below those found in the intact rats. The daily administration of 0.5 mg. of hydroxycorticosterone appeared to approach most closely the physiological dose

since this amount resulted in approximately normal numbers of eosinophils and other cell types in the marrow, as well as in normal organ weights. The degree of marrow eosinopenia depended primarily upon the amount of hydroxycorticosterone injected and secondarily upon the amount of corticosterone in the mixtures. Synergistic actions were not apparent with any of the combinations.

In most cases the effects of adrenalectomy and adrenal steroids upon marrow lymphocytes paralleled those noted for eosinophils. Thus, following adrenalectomy there was a tendency for lymphocytic concentrations to rise while treatment with adrenal steroids caused a marked lowering of their numbers. Again, the larger doses of hydroxycorticosterone were most effective in this lymphopenic action. No synergistic or antagonistic actions between the steroids were noted.

The unclassified cell category of Table 2 consisted, in great part, of disrupted cells. The concentrations of these cells displayed statistically significant fluctuations. Following adrenalectomy (Group 2) their numbers were increased while with steroid administration (Groups 4, 5, 6, 7, 8 and 10) their numbers underwent a diminution.

To summarize the effects of adrenal steroids upon marrow: these agents when injected in high dosages increased the concentrations of nucleated as well as non-nucleated erythroid cells. Eosinopenic and lymphopenic activity appeared to reside primarily in hydroxycorticosterone and corticosterone, the former being the more potent. No evidence of synergism or antagonism in the mixtures tested was seen.

Studies of bone marrow sections confirmed the quantitative cellular findings. Following adrenalectomy, the marrow appeared to have decreased areas of nucleated red blood cells and contained numerous eosinophils. After injections of adrenal steroids, the marrow displayed numerous nests of developing erythroid elements; eosinophils were scarce. With the routine techniques used, it was not possible to delineate the lymphocytic elements within sections. With high doses of hydroxycorticosterone non-nucleated erythroid cells congested the bone marrow. Grossly, the marrow was reddened in contrast to the pale marrow of the adrenalectomized rats receiving only saline injections. The marrows of the hydroxycorticosterone-treated rats were of looser consistency than those of control animals and could be drawn up more easily into a pipette.

No significant change in any of the organ weights occurred following adrenalectomy (Group 2 of Table 3). Treatment with 0.5 mg. hydroxycorticosterone daily tended to increase body weight. Mixtures containing either a small quantity of hydroxycorticosterone or no hydroxycorticosterone at all did not depress body weight (Groups 7, 8 and 9), while hydroxycorticosterone in doses higher than 0.5 mg. daily induced marked decreases in body weight (Groups 4, 5, 6, 10 and 11). With the present techniques, it was not possible to demonstrate statistically significant dif-

TABLE 3. EFFECTS OF ADRENALECTOMY AND REPLACEMENT TREATMENT ON BODY AND ORGAN WEIGHTS

(Means ± standard errors)

Group	Change in body wt. (gms.)	Bone marrow per femur (mg.)	Thymus (mg.)	Spleen (mg.)
1	0 ± 2	70.8 ± 3.5	254 ± 8	980 ± 51
2	+ 2 ± 2	73.0 ± 3.8	315 ± 15†	1109 ± 95
3	+ 9 ± 2*	79.0 ± 3.5	256 ± 14†	1317 ± 148
4	− 5 ± 3*	83.5 ± 4.0	58 ± 2†	862 ± 64†
5	−26 ± 2†	81.5 ± 3.5	64 ± 9†	721 ± 51†
6	−32 ± 3†	81.4 ± 3.9	44 ± 4†	521 ± 55†
7	+ 5 ± 2	76.3 ± 3.4	132 ± 13†	1048 ± 59
8	+ 8 ± 4	68.9 ± 4.0	171 ± 14†	1119 ± 75
9	+ 4 ± 3	79.0 ± 2.2	134 ± 7†	954 ± 87
10	+16 ± 2†	80.7 ± 1.8	25 ± 4†	755 ± 59†
11	−17 ± 2†	73.5 ± 4.2	87 ± 6†	836 ± 91†

a Group 1 served as the control for Group 2. Group 2 is the control for Group 3–11.
* $p = <0.05$ but >0.01.
† $p = <0.01$.

ferences in the weight of femoral marrow in the control and experimental groups. Because of this, calculation of the total numbers of marrow cells per femur in the various groups revealed, in general, the same types of changes already mentioned for the cell concentrations. The behavior of the thymus as a sensitive indicator of adrenal function was confirmed again in these experiments. Following adrenalectomy, thymic weights increased significantly. All the steroids, used singly or in combinations, exerted marked thymolytic activity. In many cases the thymus was reduced to a shriveled mass of tissue containing a few lymph nodes. The potency of the mixtures was again a function of their hydroxycorticosterone content and secondarily of the amount of corticosterone present. Spleen weight changes paralleled, but were not as sensitively affected as those for the thymus. The rise in spleen weight following adrenalectomy was not significant, but various steroid treatments (Groups 4, 5, 6, 10 and 11) effected decreases in splenic weights.

DISCUSSION

The cellular changes in the marrow seen after adrenalectomy and replacement therapy are more striking and, in general, precede those which occur in the peripheral blood. Thus it has been shown (Crafts, 1941; Gordon, Piliero and Landau, 1951; Van Dyke, Contopoulos, Williams, Simpson, Lawrence and Evans, 1954) that adrenalectomy in the salt-maintained rat results in a mild anemia. Adrenal cortical extracts and cortisone prevent the anemia of adrenalectomy in rats (Gordon, Piliero and Landau, 1951) and desoxycorticosterone increases the reticulocyte and red blood cell counts in the guinea pig (Cicconardi and Lorusso, 1946). Repeated injections of ACTH evoke increases in the total circulating red blood cell volume of normal as well as hypophysectomized rats (Garcia,

Van Dyke, Huff, Elmlinger and Oda, 1951). A rise in the total amount of hemoglobin and in blood volume has also been reported in hypophysectomized rats following ACTH injections (Gemzell and Sjöstrand, 1954).

Although the blood eosinopenia resulting from stimulation of the pituitary-adrenal axis has been confirmed, repeatedly the bone marrow is believed by some (Essellier, Jeanneret and Morandi, 1954) not to reflect this peripheral effect. Thus, no significant decreases in marrow eosinophils have been reported following short term treatment with ACTH in humans (Essellier and Wagner, 1952) or in guinea pigs (Gross, 1954) and with adrenal cortical extract (Durgin and Meyer, 1951) or cortisone in mice (Quittner, Wald, Sussman and Antopol, 1951). On the other hand, Gordon, Piliero and Landau (1951) found that marrow eosinopenia followed chronic treatment with cortisone in rats and Hayes and Baker (1952) obtained similar results with repeated injections of adrenal cortical extract. The experiments of Robbins, Cooper and Alt (1955) also reveal that rats receiving ACTH for 20 days manifest a relative marrow eosinopenia. The present work demonstrates, on a quantitative basis, the potent eosinopenic effects of hydroxycorticosterone and corticosterone in bone marrow. It remains to be determined whether the marrow eosinopenia results from 1) increased release of eosinophils to the circulation, 2) an inhibition of their formation, 3) their increased destruction *in situ* or a combination of these possibilities.

The depressive action of adrenal steroids upon circulating lymphocytes and lymphoid organs is well documented (Dougherty and Dougherty, 1953). However, the reduction in marrow lymphocytes following replacement treatment seen in the present experiments is not in accord with the findings of others. The failure of Yoffey *et al.* (1954) to note a lymphocytopenia in the marrow of guinea pigs following injections of either cortisone, hydroxycorticosterone or Kendall's Compound A may be due to the general refractoriness of this species to this type of treatment. Thus these workers noted that peripheral lymphocytes showed little response to cortisone and there appeared to be a slight increase in lymphocyte numbers in the experiments with Compound A. It is possible that larger doses of steroids would yield results similar to those reported herein for rats.

Marrow cells of the unclassified category in the myelograms include cells disrupted in the smears beyond recognition. Nucleated erythrocytes are generally less fragile than leukocytes. The steroid-treated rats displayed increased numbers of red blood cell precursors as well as decreased numbers of unclassified cells. The presence of fewer disrupted cells, therefore, may be the result of the shifting of the marrow, by the steroids, to a more resistant (nucleated erythroid) cell population.

The importance of dosage as a factor in evaluating the effects of adrenal steroids on blood formation must be emphasized. Treatment with 0.5 to 2.0 mg. hydroxycorticosterone daily for 7 days restored bone marrow

values in the adrenalectomized rat to approximately normal levels. On the other hand, 4.0 and 6.0 mg. doses decreased the numbers of nucleated bone marrow cells. It is of interest that there was an apparent stimulatory action upon the development of the nucleated red blood cell series of the marrow even with doses of steroids that caused severe marrow eosinopenia and lymphocytopenia.

The mechanisms by which adrenal steroids stimulate erythropoiesis are as yet unknown. Hoagland, Bergen, Slocombe and Hunt (1953) found that the amount of blood flowing through the head of rats was reduced by adrenalectomy and returned to normal with lipoadrenal extract. If similar changes occur in the blood flow through the marrow, the possibility should be examined that some hemopoietic factors (Erslev, 1953; Hodgson and Tohá, 1954; Gordon, Piliero, Kleinberg and Freedman, 1954; Borsook, Graybiel, Keighley and Windsor, 1954) or basic metabolites regulate erythropoiesis according to their rate of delivery to the marrow. An alternative possibility is that the steroids may act through their known effects on electrolyte and fluid metabolism. Thus water retention might result in an initial relative hemodilution with the possible establishment of an hypoxic state that would stimulate erythropoiesis. In consonance with this idea is the observation that desoxycorticosterone, a steroid possessing relatively weak glucocorticoid but strong mineraloid activity, exerts a marked erythropoietic action (Cicconardi and Lorusso, 1946; Fruhman and Gordon, 1954).

SUMMARY

Employing a rapid, reproducible means for estimating total cellular numbers in the bone marrow, it is found that adrenalectomy in the salt-maintained rat results in decreased concentrations of erythroid and increased concentrations of eosinophilic cells. Hydroxycorticosterone and corticosterone, alone or in combination with desoxycorticosterone, stimulate erythropoiesis in the adrenalectomized rat. Hydroxycorticosterone and corticosterone exert both eosinopenic and lymphopenic actions upon bone marrow. The erythropoietic action of adrenal steroids may be a consequence of improved circulation which involves delivery of essential nutrients to the blood forming organs or it may be the result of an hypoxic state due to initial water retention.

REFERENCES

Aschkenasy, A.: Maladies des Globules Rouges et Désordres Endocriniens. G. Doin et Cie. Paris. 1952.

Baker, B. L. and D. J. Ingle: *Endocrinology* **43**: 422. 1948.

Betz, E. H.: *Rev. d'Hématologie* **8**: 489. 1953.

Borsook, H., A. Graybiel, G. Keighley and E. Windsor: *Blood* **9**: 734. 1954.

Cavallero, C. and C. Braccini: *Proc. Soc. Exptl. Biol. Med.* **78**: 141. 1951.

Cicconardi, A. and G. Lorusso: *Bol. Soc. Ital. Biol. Sperimentale* **22**: 41. 1946.

CRAFTS, R. C.: *Endocrinology* **29**: 596. 1941.
DAUGHADAY, W. H., R. H. WILLIAMS AND G. A. DALAND: *Blood* **3**: 1342. 1948.
DOUGHERTY, T. F. AND J. H. DOUGHERTY: *Ann. Rev. Physiol.* **15**: 195. 1953.
DURGIN, M. L. AND R. K. MEYER: *Endocrinology* **48**: 518. 1951.
ERSLEV, A.: *Blood* **8**: 349. 1953.
ESSELLIER, A. F., R. L. JEANNERET AND L. MORANDI: *Blood* **9**: 1055. 1954.
ESSELLIER, A. F. AND K. F. WAGNER: *Klin. Wchnschr.* **30**: 705. 1952.
FRUHMAN, G. J. AND A. S. GORDON: *Anat. Rec.* (abstract) **120**: 780. 1954.
FRUHMAN, G. J. AND A. S. GORDON: *Proc. Soc. Exptl. Biol. Med.* **88**: 130. 1955.
GARCIA, J. F., D. C. VAN DYKE, R. L. HUFF, P. J. ELMLINGER AND J. M. ODA: *Proc. Soc. Exptl. Biol. Med.* **76**: 707. 1951.
GEMZELL, C. A. AND T. SJÖSTRAND: *Acta endocrinol.* **16**: 6. 1954.
GORDON, A. S.: *Rec. Prog. Horm. Res.* Academic Press, N. Y. **10**: 339. 1954.
GORDON, A. S.: *Ann. N. Y. Acad. Sci.* **59**: 907. 1955.
GORDON, A. S. AND H. A. CHARIPPER: *Ann. N. Y. Acad. Sci.* **48**: 615. 1947.
GORDON, A. S., S. J. PILIERO, W. KLEINBERG AND H. H. FREEDMAN: *Proc. Soc. Exptl. Biol. Med.* **86**: 255. 1954.
GORDON, A. S., S. J. PILIERO AND D. LANDAU: *Endocrinology* **49**: 497. 1951.
GRANT, W. C. AND W. S. ROOT: *Physiol. Rev.* **32**: 449. 1952.
GROSS, R.: *Rev. d'Hématologie.* **9**: 504. 1954.
HAYES, M. A. AND B. L. BAKER: *U. Mich. Med. Bull.* **18**: 109. 1952.
HOAGLAND, R., J. R. BERGEN, A. SLOCOMBE AND C. HUNT: *Ann. N. Y. Acad. Sci.* **56**: 659. 1953.
HODGSON, G. AND J. TOHÁ: *Blood* **9**: 299. 1954.
HUDSON, G., G. HERDAN AND J. M. YOFFEY: *Brit. Med. J.* **1**: 999. 1952.
QUITTNER, H., N. WALD, L. N. SUSSMAN AND W. ANTOPOL: *Blood* **6**: 513. 1951.
ROBBINS, G. P., J. A. D. COOPER AND H. L. ALT: *Endocrinology* **56**: 161. 1955.
STEIN, K. F. AND C. MARTIN: *Proc. Soc. Exptl. Biol. Med.* **78**: 513. 1951.
SWINGLE, W. W. AND W. KLEINBERG: *Ann. Rev. Physiol.* **17**: 367. 1955.
THIERSCH, J. B., L. CONROY, A. R. STEVENS AND C. A. FINCH: *J. Lab. and Clin. Med.* **40**: 174. 1952.
VAN DYKE, D. C., A. N. CONTOPOULOS, B. S. WILLIAMS, M. E. SIMPSON, J. H. LAWRENCE AND H. M. EVANS: *Acta haemat.* **11**: 203. 1954.
YOFFEY, J. M., R. J. ANCILL, J. A. G. HOLT, B. OWENSMITH AND G. HERDAN: *J. Anat.* **88**: 115. 1954.

IDENTITY OF THE ARGYROPHILIC CELLS IN THE PARS DISTALIS OF THE RAT'S PITUITARY GLAND[1]

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THE McManus (1946) periodic acid-Schiff (PAS) technique and the Gomori (1950) aldehyde fuchsin stain have been employed successfully in demonstrating and elucidating the histophysiology of 3 types of pituitary gland basophils (Halmi, 1950; Pearse, 1952; Purves and Griesbach, 1955). Among other cytological and histochemical methods applied to this gland, several silver impregnation methods (Bodian protargol, Gros-Schultz, Masson) will selectively demonstrate certain cells in the pars distallis (Hamperl, 1932; Biondi, 1936; Morita, 1937; Popoff, 1943; Dawson and Barnett, 1944; Hellweg, 1951; Knigge, 1955a). The identity of the cell which exhibits this property of reducing silver has not been definitely established. Popoff (1943) suggested that argyrophilic cells represented both acidophils and basophils in a resting or non-secretory phase. Hellweg (1951), applying the protargol method to human glands, demonstrated several types of argyrophilic cells on the basis of cell size and the number and size of blackened cytoplasmic granules. She concluded that silver staining cells most closely resemble the δ, ϵ, and undifferentiated cells of Romeis (1940). Results of Knigge (1955a) indicate that argyrophilic cells are not acidophils, while the evidence with respect to the basophils is not conclusive (Dawson and Barnett, 1944; Knigge, 1955a).

The present experiments were designed to identify silver staining cells by comparing their response with changes produced in the basophils in pituitary glands of rats under experimental conditions which result in known changes in the latter cell type.

MATERIAL AND METHODS

Equal numbers (10) of male and female Sprague-Dawley rats, weighing approximately 200 gm., were subjected for 10 or 21 days to the following experimental conditions: (a) castration; (b) thyroidectomy; (c) 0.01% methylthiouracil incorporated in the drinking water; (d) 5 μg. DL-thyroxine daily; (e) 250 μg. of testosterone propionate in peanut oil daily; (f) 5 μg. of diethylstilbestrol in peanut oil daily. Untreated animals were used

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as controls for groups *a* through *d*, while controls for groups *e* and *f* received an equal amount of peanut oil (0.1 ml.) per day. The pituitary glands of 3 to 6 pregnant animals were obtained at 3, 6, 15 and 21 days of pregnancy. Similar numbers of lactating females were sacrificed after 3, 10 and 21 days.

At autopsy, the pituitary glands of several animals in each group were bisected, the halves being fixed in Bouin's fluid and formalin-alcohol-acetic acid (FAA) respectively. Those halves fixed in Bouin's fluid were cut at 5μ and stained with aldehyde fuchsin (AF) and the Masson trichrome method. The remaining glands, fixed in FAA, were cut serially at 5 μ and stained by the Bodian (1937) protargol method. Pituitaries of control and experimental animals were stained simultaneously in the protargol, subsequent treatment with gold chloride and oxalic acid being omitted.

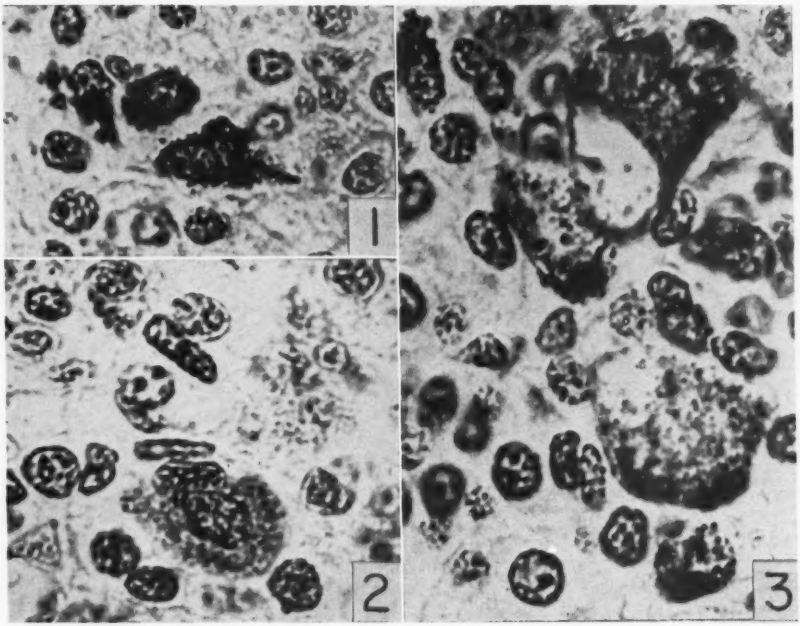
In view of the fact that "argyrophilic" cells are present in other tissues also, the specificity of the response of pituitary argyrophilic cells to the present experimental conditions was tested by preparing and staining with protargol sections of the duodenal of all the rats used.

RESULTS

Argyrophilic cells in the normal gland. Following staining by the protargol method, certain cells in the pars distalis of the pituitary gland are demonstrated by an intense blackening of their cytoplasmic granules (Fig. 1). These argyrophilic cells are present in the male gland in large numbers in that portion of each lobe which borders on the pars intermedia, an area referred to as the "sex zone" by Purves and Griesbach (1951). They are particularily concentrated anteriorly near the junction of the pars distalis with the pars intermedia and occasional ones are observed in the latter area (Figs. 6, 11). In the remainder of the pars distalis, argyrophilic cells are distributed almost uniformly in the peripheral and central parts of each lobe. Although it is not apparent in all sections, argyrophilic cells are characteristically grouped along the large portal vessels and sinusoids (Figs. 6, 11). The relative percentage of silver staining cells has been calculated to be approximately 6–7% (Knigge, 1955a). Argyrophilic cells vary considerably in shape, the majority being oval or elongate; angular forms and cells with long processes are also present (Figs. 1, 8). The blackened granules present in the majority of argyrophilic cells are densely packed and distributed uniformly in the cytoplasm (Fig. 1). Occasionally a clear juxtanuclear area is seen, representing the negative image of the Golgi apparatus. In a relatively small number of cells conspicuously smaller granules are sparsely distributed in lightly stained cytoplasm, imparting a brownish color to the cell (Fig. 2).

In contrast to the abundant numbers of argyrophilic cells present in the male pituitary gland, glands of females of this age contain relatively few (Fig. 4; Table 1). Those present are distributed without notable localization in each lobe and are similar to silver staining cells observed in the male gland.

Effect of castration or thyroidectomy. Ten days after gonadectomy of the



FIGS. 1–3. Silver staining cells in the pituitary glands of control male rats. All glands fixed in FAA and stained with protargol. ×1100.

FIG. 1. Several typical silver staining cells containing argyrophilic granules which are large and densely packed. One cell exhibits a process containing a single row of granules.

FIG. 2. A silver staining cell is present whose cytoplasm is lightly stained and which contains only a relatively few blackened granules.

FIG. 3. Several large vacuolated silver staining cells. Such cells are very infrequent in the pituitaries of control or treated animals. The blackened granules, which vary somewhat in size, are generally round or slightly oval and appear to be more concentrated along one border or at one pole of the cell.

male, the number of argyrophilic cells is markedly reduced. After 21 days, silver staining cells are virtually absent except for an occasional, lightly staining cell (Fig. 9). A similar decline is observed in the pituitaries of ovariectomized animals, with the disappearance complete at 10 days after operation. Glands stained with AF-Masson reveal a characteristic hypertrophy of the light green-staining delta basophils, with no significant alteration in the AF-positive beta cells or in the acidophils. The number and appearance of argyrophilic cells in the pituitary glands of male and female rats thyroidectomized or fed methylthiouracil for 10 or 21 days are not significantly different from those in control glands. In pituitaries of thyroidectomized animals, a degranulation of acidophils, almost complete absence of AF-positive beta cells, and hypertrophy and vacuolation of delta cells is present in AF-Masson preparations. These vacuolated delta cells

TABLE 1. COMPARISON OF ARGYROPHILIC CELLS WITH THE PAS-STAINING "GONADOTROPHS" AND "THYROTROPHS" IN THE PARS DISTALIS OF THE RATS' PITUITARY GLAND

Treatment	Argyrophilic cells	PAS-staining of basophils[1]	
		"Gonadotrophs"	"Thyrotrophs"
None	Few and scattered in female gland; many, intensely stained and concentrated along border of p. intermedia in male gland	All faintly stained in female; in male "peripheral" cells intensely stained, "central" ones light	Lightly stained; centrally located
Castration	Almost complete disappearance	Initial decrease; then progressive increase to intense staining	No change
Thyroidectomy	No significant effect	No change	Initial decrease; then progressive increase to intense staining
Testosterone propionate	Slight increase in male; very marked increase in female	Increase in "peripheral" and "central" cells; then later decrease staining of "central" cells	No change
Stilbestrol	Almost complete disappearance	Progressive decrease	No change to slight decrease
Thyroxine	Slight decrease in male gland	No change or slight decrease	Loss of staining
Pregnancy	Very marked increase	Marked increase	Increase

[1] Data from Purves and Griesbach (1951, 1954, 1955).

(thyroidectomy cells) are believed to arise from beta basophils which lose their affinity for aldehyde fuchsin (Halmi, 1952).

Effect of hormone administration. Administration of 5 μg. of DL thyroxine for 10 or 21 days produces a slight decrease in the number of argyrophilic cells, this decline being apparent only in the male gland and in that area bordering on the pars intermedia where argyrophilic cells are normally most concentrated (Fig. 11). A slight but consistent decline in number of AF-staining beta cells is the only notable change in AF-Masson sections.

In both sexes, administration of 5 μg. of diethyl-stilbestrol for 10 days results in a virtually complete disappearance of argyrophilic cells (Fig. 10), this condition being unchanged after 21 days of treatment. Treatment with testosterone propionate (250 μg. per day) leads to an increase in

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EXPLANATION OF FIGURES 4–7

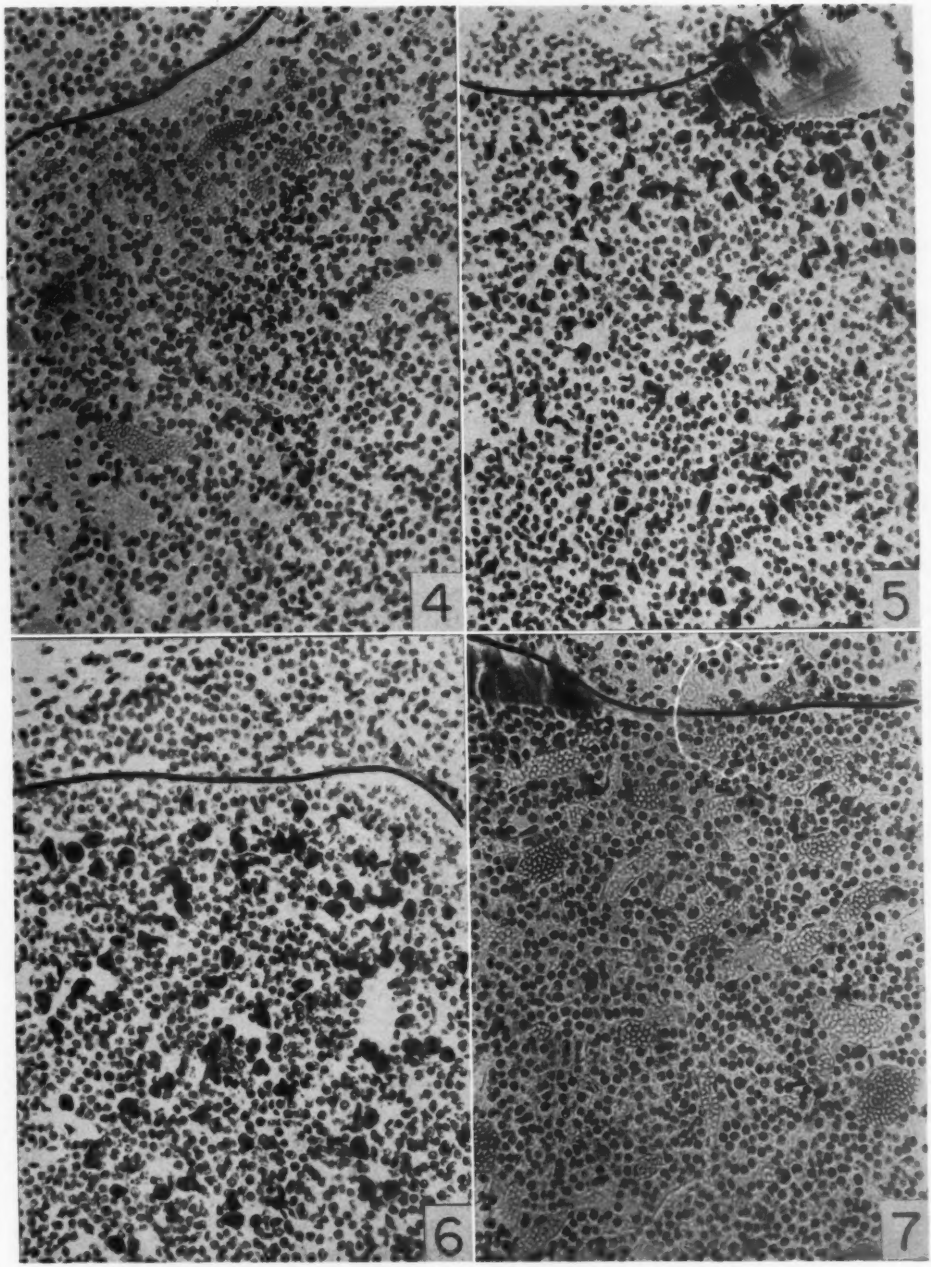
FIGS. 4–7. Pituitary glands from control and experimental female rats. In all photographs, a black line demarcates the boundary between the pars intermedia and pars distalis. The lower margin of each photograph is the approximate center of the section of pars distalis. FAA, Protargol. ×235.

FIG. 4. Control. Only a few, scattered silver staining cells are present. The nuclei of all cells are stained with silver also.

FIG. 5. Ten days of treatment with testosterone propionate (250 μg. per day) evokes a marked increase in the number of argyrophilic cells.

FIG. 6. On the tenth day of pregnancy, the pituitary contains an abundant number of silver staining cells which appear somewhat larger than those seen in control or testosterone-treated animals. The location of argyrophilic cells along the sinusoids of the pars distalis is apparent, while several small argyrophilic are present in the pars intermedia at the right margin of the photograph.

FIG. 7. Twenty-one days after parturition, glands of lactating animals contain virtually no silver staining cells.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number of argyrophilic cells of the pituitary without notable difference between animals treated for 10 or 21 days (Fig. 5). The pituitaries of testosterone-treated females contain greater numbers of argyrophilic cells than are present in glands of control or testosterone-treated males.

Examination of AF-Masson stained sections indicate that neither diethyl-stilbestrol nor testosterone propionate produced significant changes in the AF-positive beta basophils in male or female pituitaries. The appearance of the delta basophils after estrogen treatment suggests a degranulation and reduction in number, although this was not as marked as is usually seen after longer treatment with greater amounts of this hormone (Halmi, 1951). After 21 days of treatment with testosterone propionate, delta basophils exhibit some hypertrophy and vacuolation.

Effect of pregnancy and lactation. The pituitary glands of animals pregnant for 3, 6, 15 and 21 days reveal a striking increase in number of intensely stained argyrophilic cells (Fig. 6). Although the differences are not marked, the number of silver staining cells appear greater at 15 and 21 than at 3 or 6 days of pregnancy. Three days after parturition, the pituitary glands contains significantly fewer argyrophilic cells, although they are still present in numbers greater than in the glands of control, nonlactating females. At 10 and 21 days of lactation, the impression is gained that the number of argyrophilic cells is reduced below that observed in control glands (Fig. 7).

Effect on argyrophilic cells of the duodenum. The distribution, appearance and number of argyrophilic cells in the duodenal mucosa of the control rats in the present study are similar to that observed by Baker and Bridgman (1954). While a more detailed account of the effects of the present experimental conditions on the duodenal argyrophilic cells will be presented elsewhere, several effects can be noted in order to compare the response of the argyrophilic cells in the pituitary gland with those in the duodena of similarily treated animals. Testosterone propionate, which elicits a marked increase in number of pituitary argyrophilic cells, has no

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EXPLANATION OF FIGURES 8–11

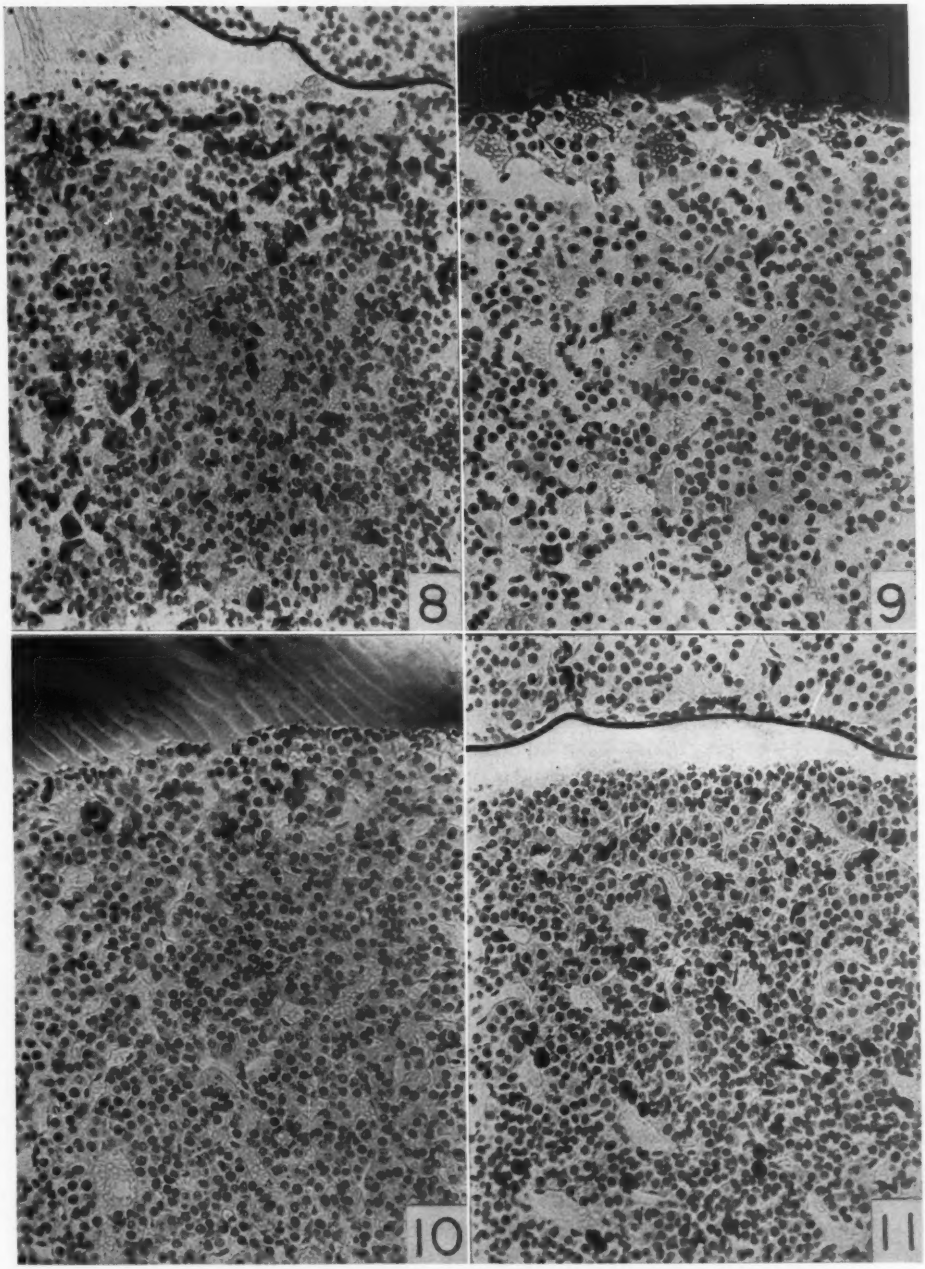
FIG. 8–11. Pituitary glands from control and experimental male rats. FAA, Protargol. ×235.

FIG. 8. Control. Silver staining cells, both oval and angular in shape, are most concentrated along the border of the pars distalis and distributed almost uniformly throughout the remainder of the lobe.

FIG. 9. Twenty-one days after castration, only a few scattered silver staining cells are present.

FIG. 10. Ten days of treatment with diethyl-stilbestrol (5 μg. per day) leads to a complete disappearance of silver staining cells.

FIG. 11. Twenty-one days of treatment with thyroxine (5 μg. per day) produces some decrease in the number of silver staining cells in the zone adjacent to the pars intermedia.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notable effect on those of the duodenum. Thyroxine does not affect significantly the pituitary argyrophilic cells, but increases the number of silver staining cells of the duodenal mucosa.

Correlation with PAS-basophils. PAS-staining basophils have been studied in the rat under experimental conditions similar to those of the present study, and Purves and Griesbach (1955) differentiated them into a group of "gonadotrophs" and a group of "thyrotrophs." The latter are the AF-positive beta cells of Halmi. A comparison of the response of cells demonstrated by the PAS-method and by the protargol silver technique is presented in Table 1.

DISCUSSION

Argyrophilic cells in the rat's pituitary gland, demonstrated with protargol silver, exhibit reactions different from similarily staining cells in the duodenal mucosa and appear to reflect the activity of a specific pituitary cell type. AF-positive beta cells (thyrotrophs) appear to be unrelated to the silver staining cells because those conditions which alter the number or staining properties of the thyrotrophs have little or no effect on argyrophilic cells. Results of the present study indicate that argyrophilic cells respond similarly and may be identical with the PAS-staining basophils referred to as gonadotrophs by Purves and Griesbach. Both argyrophilic cells and gonadotrophs are: (1) spatially distributed in approximately similar patterns and are characteristically located along portal vessels and sinusoids; (2) present in greater numbers in the male gland; (3) increased markedly in pregnancy or after administration of testosterone propionate; (4) decreased by estrogen treatment, and (5) only slightly affected by thyroidectomy or treatment with thiouracil or thyroxine.

Following castration there was a prompt and almost complete disappearance of argyrophilic cells which was maintained for the duration of the experiment (21 days). This differs somewhat from the response of gonadotrophs as determined by staining with PAS. Although there is an initial decline in their PAS staining after castration, the gonadotrophs subsequently exhibit a progressive increase in staining, and become intensely PAS positive several weeks after castration (Purves and Griesbach, 1955). The solution of this apparent discrepancy may lie in recent electron microscopy studies of Farquhar and Rinehart (1954a, 1954b), which described the effects of castration and thyroidectomy on the pituitary gonadotrophs and thyrotrophs, respectively. The cytoplasm of both cells contains granules and vesicles, the PAS staining reaction being related to the granules and vesicles of the gonadotroph but only to the vesicles of the thyrotroph. Granules in the latter cell are electively stained with AF. Thyroidectomy leads to a prompt and almost complete disappearance of the granules in thyrotrophs, which correlates well with an equally rapid decline in AF-staining of these cells. Following gonadectomy, the granule

content of gonadotrophs declines while cytoplasmic vesicles increase in number and size. Since both granules and vesicles of the gonadotroph are PAS-positive, the initial decline and subsequent increase in PAS staining noted by Purves and Griesbach (1855) may be related to the loss of cytoplasmic granules and increase in vesicles as demonstrated by the electron microscope. In view of the fact that argyrophilic cells do not reappear after castration, it follows that, if all or some of these cells are identical with gonadotrophs, the property of argyrophilia in this case is related to the cytoplasmic granules only. Further specificity of this staining may be implied by noting that other PAS-positive structures in the pituitary gland were not stained with silver. These include the cytoplasmic vesicles of thyrotrophs (in normal or thyroidectomized animals), elastic fibers and elastic laminae of blood vessels, and basement membranes.

Evidence from other investigations lend support also to the present conclusion that silver staining cells may represent gonadotrophic basophils. Argyrophilic cells are observed first in the pituitary glands of rats approximately 8–10 days of age (Knigge, 1955b), at which time PAS (glycoprotein) granules appear in the gonadotrophs (Siperstein *et al.*, 1954). In the rabbit, Popoff (1943) noted that argyrophilic cells were virtually absent during the months of December and January, at which time the lowest gonadotrophin content was noted by Friedman and Friedman (1939). A lowered gonadotrophin content in the pituitary glands of aged humans may be reflected also by the decline in argyrophilic cells observed by Popoff (1942) and Hellweg (1952). The marked increase in number of argyrophilic cells observed in the rat's pituitary during pregnancy was seen also in the pituitaries of pregnant rabbits (Morita, 1937). The gonadotrophin content of the pregnant rabbits' pituitary may be greater than normal according to Hill (1934), although there are conflicting reports with respect to pituitary gonadotrophin content during pregnancy of the rabbit as well as other species (Evans and Simpson, 1929; Zeiner, 1952).

Purves and Greisbach (1954, 1955) were able to differentiate two PAS-positive gonadotrophs by correlating their response to testosterone propionate treatment or to castration with the changes which occur in the FSH and LH components of pituitary gonadotrophin under these conditions (Greep and Jones, 1950). Thus, for example, treatment with 250 μg. of testosterone propionate for 10 days elicited a marked increase in the "peripheral" and "central" gonadotrophs of the female gland. After 28 days, the central gonadotrophs regressed (PAS-negative) while the peripheral ones remained PAS-positive. The gonadotrophin of the 28-day castrate animal's pituitary is largely FSH, suggesting that the peripheral gonadotrophs are the source of this hormone. In the present study there were no notable cytological differences between peripheral or centrally located silver staining cells. There were, likewise, no differences with respect to number of argyrophilic cells between the glands of females

treated with testosterone propionate for 10 or 21 days. Modification of the protargol technique as applied to the pituitary gland, presently under investigation, may permit resolution of 2 types of silver staining gonadotrophs. It is possible, however, that only one component of the gonadotrophin complex is reflected by this technique.

SUMMARY

Silver staining cells of the rat's pituitary gland, demonstrated with the protargol silver method, are much more numerous in the male gland, exhibit a characteristic distribution in the pars distalis, and are situated frequently along the portal vessels and sinusoids. Castration or estrogen treatment (5 μg. of diethyestilbestrol per day) diminish their number markedly in both sexes. Testosterone propionate (250 μg. per day) or pregnancy elicit a striking increase in argyrophilic cells. Thyroidectomy, methylthiouracil or thyroxine (5 μg. per day) affect these cells slightly or not at all.

These results indicate that silver staining cells respond similarily and may be identical with the PAS-positive gonadotrophs of Purves and Griesbach. On the basis of the changes which occur in the argyrophilic and PAS staining properties of the gonadotrophs after castration, it is suggested that only their cytoplasmic granules are stained with silver.

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REFERENCES

BAKER, B. L. AND R. M. BRIDGMAN: *Am. J. Anat.* **94:** 363. 1954.
BIONDI, G.: *Arch. f. Psychiatr.* **104:** 431. 1936.
BODIAN, D.: *Anat. Rec.* **69:** 153. 1937.
DAWSON, A. B. AND J. BARNETT: *Stain Tech.* **19:** 115. 1944.
EVANS, H. M. AND M. E. SIMPSON: *Am. J. Physiol.* **89:** 379. 1929.
FARQUHAR, M. G. AND J. F. RINEHART: *Endocrinology* **54:** 516. 1954a.
FARQUHAR, M. G. AND J. F. RINEHART: *Endocrinology* **55:** 857. 1954b.
FRIEDMAN, M. S. AND G. S. FRIEDMAN: *Endocrinology* **24:** 626. 1939.
GOMORI, G.: *Am. J. Clin. Path.* **20:** 665. 1950.
GREEP, R. O. AND I. C. JONES: In: *Recent Progress in Hormone Research.* Academic Press, Inc., N. Y., **5:** 197. 1950.
HALMI, N. S. *Endocrinology* **47:** 289. 1950.
HALMI, N. S.: *Anat. Rec.* **109:** 300. 1951.
HALMI, N. S.: *Endocrinology* **50:** 140. 1952.
HAMPERL, H.: *Virchow's Arch.* **286:** 811. 1932.
HELLWEG, G.: *Zeitschr. f. Zellforsch. u. mikr. Anat.* **36:** 349. 1951.
HILL, R. T.: *J. Physiol.* **83:** 129. 1934.
KNIGGE, K. M.: *Anat. Rec.* **122:** In Press. 1955a.
KNIGGE, K. M.: Unpublished observations. 1955b.
MCMANUS, J. F. A.: *Nature*, London **158:** 202. 1946.
MORITA, S.: *Mitt. a. d. med. Akad. Kioto* **18:** 815. 1937.

POPOFF, A.: *Anat. Rec.* **87:** 1. 1943.

PEARSE, A. G. E.: In: *Ciba Foundation Colloquia on Endocrinology.* Blakiston Co., N. Y. **4:** 1. 1952.

PURVES, H. D. AND W. E. GRIESBACH: *Endocrinology* **49:** 244. 1951.

PURVES, H. D. AND W. E. GRIESBACH: *Proceedings of the University of Otago Medical School* **30:** 27. 1952.

PURVES, H. D. AND W. E. GRIESBACH: *Endocrinology* **55:** 785. 1954.

PURVES, H. D. AND W. E. GRIESBACH: *Endocrinology* **56:** 374. 1955.

ROMEIS, B.: In: *Handbuch der mikroskopischen Anatomie des Menschen.* J. Springer, Berlin. Band VI/3. 1940.

SIPERSTEIN, E., C. W. NICHOLS, JR., W. E. GRIESBACH AND I. L. CHAIKOFF: *Anat. Rec.* **118:** 593. 1954.

ZEINER, F. N.: *Anat. Rec.* **113:** 255. 1952.

THE PREPUBERAL PUBIC SYMPHYSIS AND UTERUS IN THE MOUSE: THEIR RESPONSE TO ESTROGEN AND RELAXIN[1]

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THERE is a marked sexual dimorphism of the pelvis in adult mice. The pelvis of the female mouse differs from that of the male by having smaller bones, a greater ischio-pubic angle and a more posteriorly placed pubic symphysis (Gardner, 1936). These differences develop even when mice are gonadectomized at 3 weeks of age (Crelin and Haines, 1955). Puberty in the mouse usually occurs shortly after the 6th postnatal week (Allen, 1923). Following puberty the medial ends of the female's pubic bones resorb partially at the symphysis. This does not occur in mice ovariectomized before puberty (Crelin and Haines, 1955). Pubic bone resorption occurs in both gonadectomized and intact estrogen-treated mice. (Gardner, 1936; Hall, 1950; Crelin, 1954a). During the estrous cycle in mice a slight interpubic separation occurs (Kliman *et al.*, 1952). During the first pregnancy the interpubic separation is increased greatly and the medial ends of the pubic bones undergo further resorption. The symphysial cartilage becomes flexible and swollen before it undergoes dissolution to be replaced by an interpubic ligament (Hall, 1947, 1954; Crelin, 1954a, 1954c). Changes similar to those occurring during pregnancy were produced in gonadectomized adult mice by injecting estrogen and relaxin (Hall, 1947, 1948; Trentin, 1951; Dorfman *et al.*, 1953; Kliman *et al.*, 1953a, 1953b; Crelin, 1954a, 1954b).

A search of the literature failed to reveal any studies principally concerned with the development of the mouse's pelvis. Thus, it was not known what the first sexual dimorphic changes of the pelves are or when they occur in development. Also, it was not known when in development the pubic bones and interpubic tissue first become capable of responding to effective amounts of estrogen and relaxin. Because the amounts of estrogen and relaxin necesary to produce a response at different times in development were unknown, amounts sufficient to produce a definite response in the gonadectomized adult were injected. The immature mouse's uterus responds to estrogen treatment by becoming distended with fluid (Bur-

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rows, 1949). Thus, this structure served principally as an indicator that the injected estrogen had its proper potency in the very young females, whose symphysial tissue was capable of responding only slightly or not at all.

MATERIALS AND METHODS

Seventy-three female and 75 male newborn mice of the Brown-belt strain were arranged into 6 groups. One group was sacrificed at each weekly interval from the end of the 1st to the end of the 6th postnatal week. Untreated mice of both sexes in each group served as controls. One-half of the remainder of males and females in each group were treated with estradiol benzoate alone; the other half with estradiol benzoate and relaxin. Hormonal treatment was started 5 days before the animals were sacrificed. The mice treated with estrogen alone received a subcutaneous injection containing 1 μg of estradiol benzoate in sesame oil on the 1st and 3rd day and were killed on the 5th day. The animals treated with both estradiol benzoate and relaxin received the same treatment described above except that 24 hours before necropsy each received a subcutaneous injection containing 100 G.P. units of relaxin.[2]

The mice were autopsied immediately following digital separation of the atlanto-occipital joint. The uteri were examined grossly and the middle third of the right cornua were excised for histological study. The pubic symphysis of each mouse was exposed and its flexibility determined in situ by manipulating the innominate bones with forceps. The symphysis was considered flexible only when the range of movement of the pubic bones at the symphysis was distinct. A significant interpubic separation (0.5 mm. or more) was measured directly with a caliper in a manner previously described (Crelin, 1954a). The symphyses and uteri were fixed in Bouin's fluid. Representative sections of the uteri and serial-sections of the symphyses were made and stained with hematoxylin and erythrosin.

RESULTS

Untreated Control Mice

Group I (*1 wk. C*[3] *2* ♀, *2* ♂) In both sexes a solid, inflexible bar of small-celled hyaline cartilage constituted the ventral portion of the pelvis (Fig. 6). Laterally this cartilage connected the developing pubic and ischial bones. (The central portion of the bar later becomes the interpubic tissue of the symphysis; the two lateral portions are replaced by the inferior rami of the pubic bones.) A lamina of compact cartilage cells, 3 to 4 cells wide, occupied the cranial half of the bar and extended midsagittally from the dorsal surface to about the bar's center, failing to reach the ventral surface. Only a small amount of matrix separated the lacunae. The chondrocytes were spherical and were the same size as the adjacent more widely spaced cells (Fig. 6).

Each uterus had a broad inner circular muscle layer and a thin outer longitudinal layer. The uterine lumen was small and glands were sparse. Each gland was a short, straight invagination of the epithelium with a

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[2] Releasin generously supplied by Dr. Robert L. Kroc. Warner-Chilcott Labs. Morris Plains, N. J.

[3] Control.

FIG. 1. Symphyses of estrogen- and relaxin-treated mice.

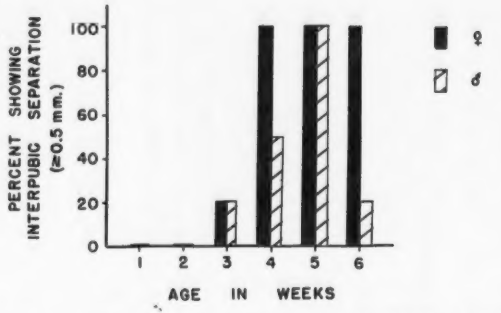


FIG. 2. Symphyses of estrogen- and relaxin-treated mice.

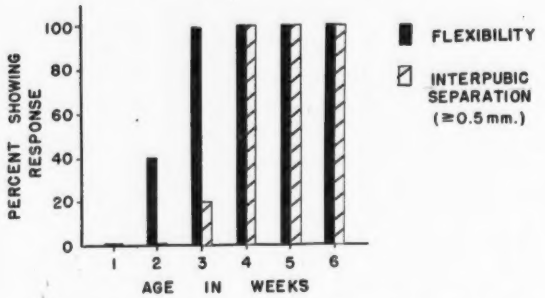


FIG. 3. Symphyses of estrogen- and relaxin-treated ♀ mice.

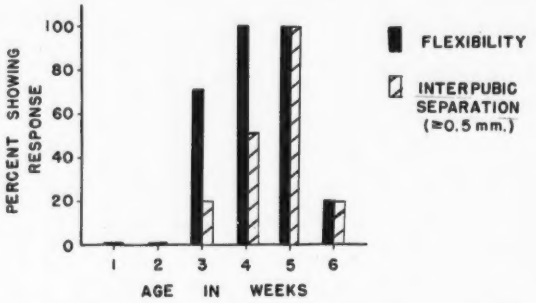


FIG. 4. Symphyses of estrogen- and relaxin-treated ♂ mice.

wide lumen. The cells of the uterine epithelium and the developing glands were tall columnar, each having an oval nucleus situated at its base (Fig. 20).

Group II (*2 wks. C 2 ♀, 2 ♂*) In both sexes the inferior rami of the pubic bones extended caudally on each side to about the middle third of the interpubic cartilage bar. Over-all growth of the inflexible interpubic cartilage

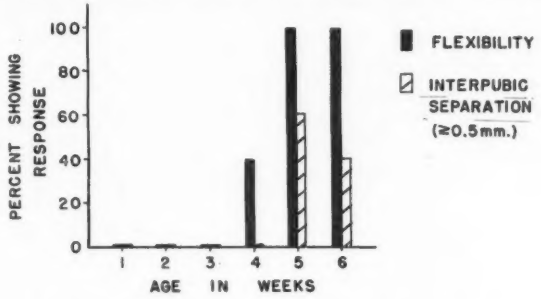
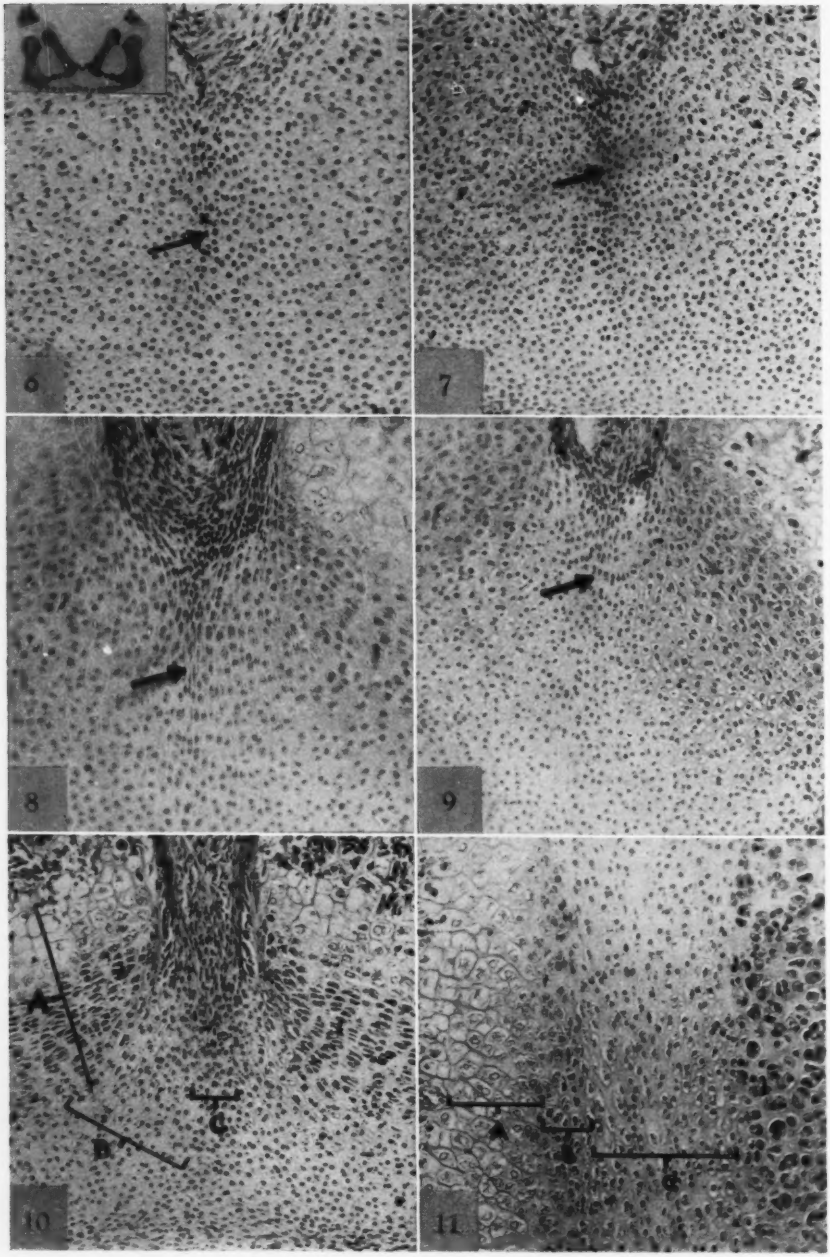


FIG. 5. Symphyses of estrogen-treated ♀ mice.

was slight in both sexes and the histological findings in the male were similar to those of the 1-week-old controls (Fig. 9). In the females the cells of the compact cartilage lamina in the midline were partially flattened; their long axes ran equally in all directions within the sagittal plane (Fig. 8). This lamina was apparently developing into fibrocartilage, for small bundles of longitudinally arranged collagen fibers were found in its matrix. As these partially flattened cells and the collagen bundles approached the thickened cranial portion of the perichondrium, which later becomes the superior pubic ligament, they gradually changed their direction to blend with the perichondrial fibroblasts and collagen bundles running in a transverse direction.

The uteri were slightly larger than those of the 1-week-old controls. An oval nucleus was situated at the base of tall columnar cells which lined straight uterine glands.

Group III (*3 wks. C 2 ♀, 2 ♂*) In both sexes the inferior rami of the pubic bones extended caudally on each side of the inflexible interpubic cartilage bar to almost its caudal border. Histologically the fibrocartilage lamina in the female appeared about the same as it did in the 2-week-old female controls except that it extended almost to the caudal border of the interpubic cartilage bar. It still did not reach the bar's ventral surface. In the male the lamina was slightly more extensive than in the 2-week-old males; however, the cells were still spherical. In both sexes the amount of longitudinally arranged collagen bundles had increased in the fibrocartilage lamina when compared with the 2-week-old controls. In both sexes, the cartilage adjacent to the intermediate fibrocartilage lamina was in the



FIGURES 6–11

form of thick articular caps of hyaline cartilage containing small spherical cells arranged somewhat into curved parallel rows which followed the contour of the laterally situated hypertrophic growth cartilage and pubic bone (Fig. 10). Thus, at the end of the 3rd postnatal week an immature but definite pubic symphysis was present.

The uteri were slightly larger than those of the 2-week-old females and had an increased number of glands. The ducts of all the glands were narrow. Their cells were tall columnar and each had a round nucleus located at the base. Some of the glands had slightly tortuous distal ends.

Group IV (4 wks. C 2 ♀, 2 ♂) In both sexes the inferior rami of the pubic bones completely formed the lateral walls of the inflexible pubic symphysis. In the male the articular caps of hyaline cartilage were still quite thick but relatively reduced in size when compared with those of the 3-week-old males. Also, the intermediate lamina of fibrocartilage was broad and midsagittally extended completely through the interpubic tissue. Its cells were compact but not flattened (Fig. 11). In the females the articular caps resembled the adult female in that they were thin and the cells compact. As in the male, the intermediate fibrocartilage lamina in the female was broad and midsagittally extended completely through the interpubic tissue. The cells of the lamina in the female were quite compact and flattened (Fig. 12). In both sexes the homogeneous fibrocartilage matrix stained more densely than the articular caps. No individual bundles of collagen fibers were seen within it.

The uteri were about twice the size of those of the 1-week-old females. The muscular wall of each was thick and the lumen small. The glands ex-

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EXPLANATION OF FIGURES 6–11

FIGS. 6–11 are coronal sections through the center of the pubic symphysis.

FIG. 6. 1-week-old ♀ control. The lamina of compact cartilage cells is shown (arrow). ×100.

Left hand corner insert is a whole mount preparation containing the pubic and ischial and portions of the ilial bones stained with alizarin red-S. Dotted lines delineate the cleared bar of hyaline cartilage constituting the ventral portion of a pelvis from an untreated 1-week-old female. ×4.

FIG. 7. 1-week-old ♀ treated with estradiol and relaxin. A complete lack of response is evident. The lamina of compact cartilage cells is shown (arrow). ×100.

FIG. 8. 2-week-old ♀ control. Flattened cells of the compact lamina are shown (arrow). ×100.

FIG. 9. 2-week-old ♂ control. Cells of the compact lamina are spherical (arrow). ×100.

FIG. 10. 3-week-old ♂ control. Collagen fibers were found in the matrix of the compact lamina. (A) hypertrophic cartilage, (B) broad articular hyaline cartilage cap, (C) fibrocartilage lamina. ×100.

FIG. 11. 4-week-old ♂ control. Cells of the compact fibrocartilage lamina are spherical. (A) hypertrophic cartilage, (B) articular cap, (C) fibrocartilage lamina. ×100.

tended through a thick submucosa to the circular muscle layer. Many glands had quite tortuous distal portions (Fig. 22).

Group V (5 wks. C 2 ♀, 2 ♂) With the exception of thinner articular caps and a narrow layer of fibrocartilage in the males' symphyses there was little change in the pelves when compared with the 4-week-old controls.

The uteri were about 3 times the size of those of the 1-week-old mice and structurally resembled those of the 4-week-old females.

Group VI (6 wks. C 2 ♀, 2 ♂) The symphyses of both sexes resembled their respective mature types: the young male and the virgin female. They were less than one-half larger in size than the 1-week-old developing symphyses. The articular caps in both sexes were thin and the fibrocartilage lamina quite narrow, resulting in a close approximation of the pubic bones. The fibrocartilage lamina of each animal contained very flattened, compact cells and a longitudinal midline cleft in the matrix of the type often found in histological sections of adult male and virgin female symphyses (Fig. 13). The symphyses were inflexible. The first signs of a sexual dimorphism of the innominate bones had appeared for the bones were wider and thicker in the male, especially the portion of the pubic bones forming the lateral walls of the symphysis. In relation to sacral levels the symphysis in the female was situated more caudally and the ischio-pubic angle was greater.

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EXPLANATION OF FIGURES 12–17

FIGS. 12–17 are coronal sections through the center of the pubic symphysis.

FIG. 12. 4-week-old ♀ control. Cells of the fibrocartilage lamina are flattened. ×100.

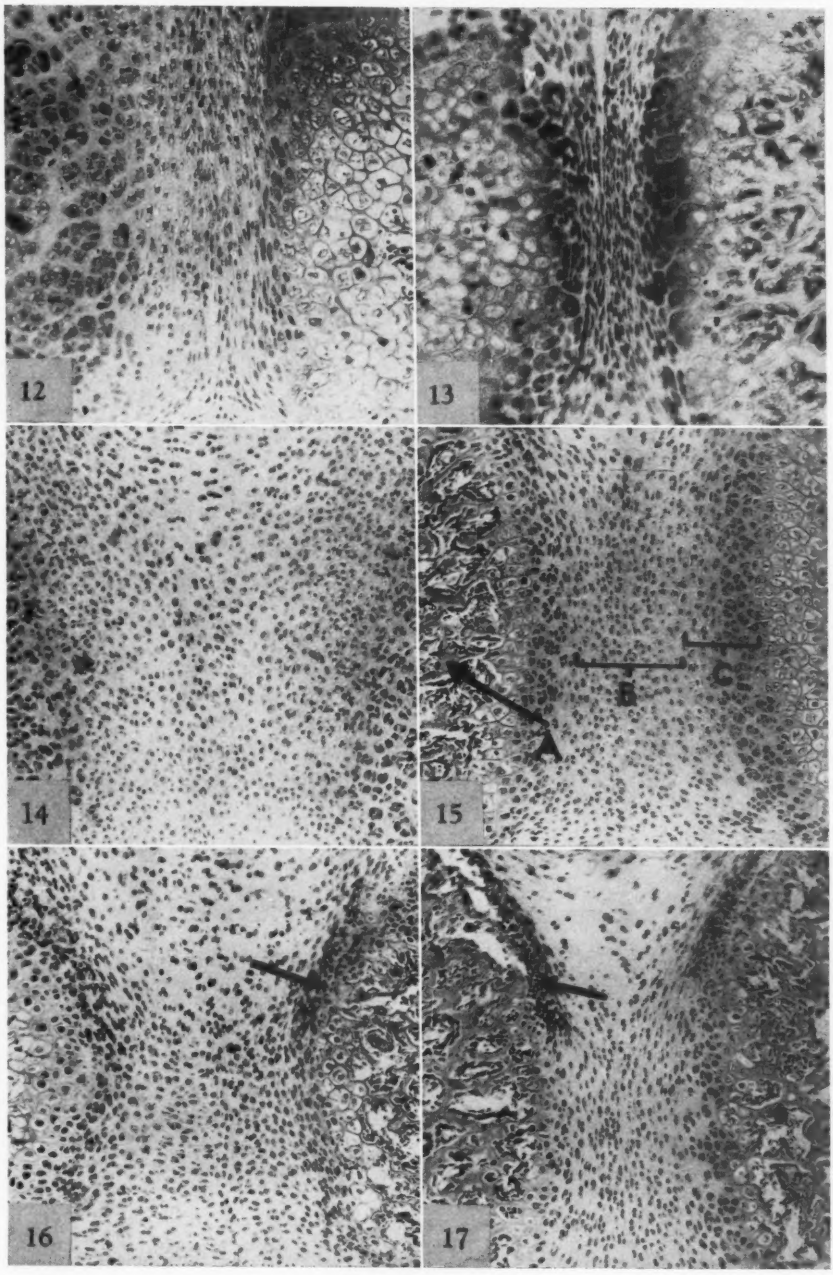
FIG. 13. 6-week-old ♀ control. The cells of the fibrocartilage lamina are markedly flattened and the matrix quite dense. ×100.

FIG. 14. 4-week-old ♀ treated with estradiol and relaxin. The matrix of the lamina and caps is swollen; being most pronounced in the cranial portion. The cartilage cells are spherical and widely spaced. Cranially many lacunae contain 2 cells. The interpubic separation was 0.7 mm. This response is similar to the maximum found in the females treated with estradiol alone. ×100.

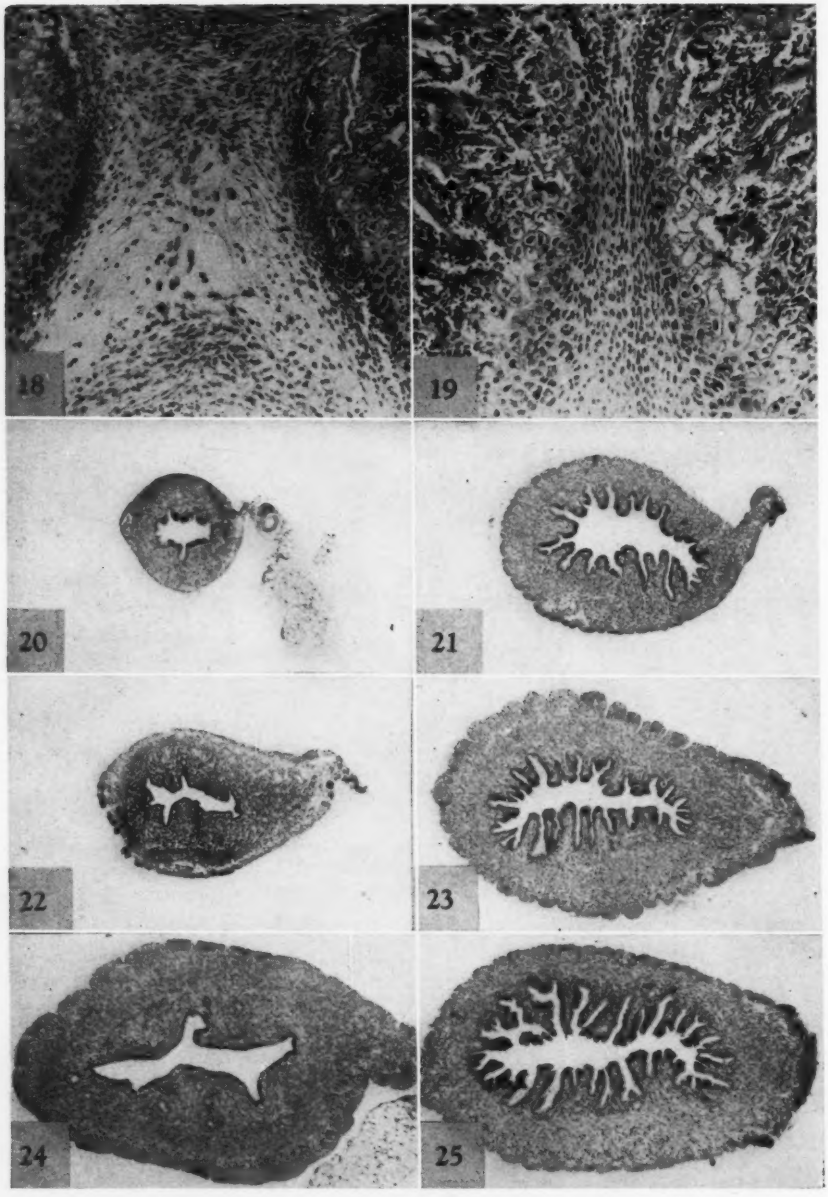
FIG. 15. 4-week-old ♂ treated with estradiol and relaxin. (A) pubic bone, (B) swollen fibrocartilage, (C) swollen articular cap. The interpubic separation was 0.5 mm. ×100.

FIG. 16. 4-week-old ♀ treated with estradiol and relaxin. The cartilage matrix in the cranial half of the symphysis is greatly swollen and contains disorganized collagenous fibers. In this area the fibrocartilage cells are dividing. The cartilage of the cranial portion of the articular caps is replaced by a dense layer of proliferating fibroblast-like cells (arrow). The interpubic separation was 1.0 mm. ×100.

FIG. 17. 5-week-old ♂ treated with estradiol and relaxin showing the maximum response of its sex to these hormones. The fibrocartilage matrix in the cranial portion of the symphysis is markedly swollen; however, the more caudal portion shows only a moderate degree of swelling. Dividing fibroblasts are widely spaced in the cranial portion and the adjacent articular caps are replaced by a layer of proliferating fibroblast-like cells forming a periosteum for the pubic bones (arrow). The interpubic separation before fixation was 0.9 mm. ×100.



FIGURES 12–17



EXPLANATION OF FIGURES 18–25

FIGS. 18 and 19 are coronal sections through the center of the pubic symphysis. Figures 20–25 are cross sections through the middle third of the right cornu of the uterus.

The uteri were about 4 times the size of those of the 1-week-old mice but still imature when compared with the uterus of the adult virgin, especially in regard to gland development (Fig. 24).

Estradiol-treated Mice

Group I (1 wk. E[4] *5 ♀, 5 ♂)* In both sexes the injected estradiol did not alter the developing pubic symphyses. Three males and 2 females had distended urinary bladders. The walls of the distended bladders were not hypertrophied. In fact, none of the distended urinary bladders of the entire experiment had thickened walls.

Each uterus was markedly distended with fluid, and when incised the uterus collapsed as the fluid escaped. The cross-sections of the collapsed uteri were slightly larger in diameter than those of the controls; however, the walls were reduced in thickness. Few mitoses were seen. The cytological detail was similar to that of the controls.

Group II (2 wks. E 5 ♀, 5 ♂) The developing symphyses resembled those of the controls. Every female and 4 of the males had greatly distended urinary bladders.

Each uterus was greatly distended with fluid to the extent that the walls were semi-transparent. The diameter of the collapsed uteri in cross-sec-

[4] Estrogen-treated.

FIG. 18. 5-week-old ♀ treated with estradiol and relaxin. Nearly all of the articular caps are replaced by a dense layer of proliferating fibroblast-like cells. The cranial half of the fibrocartilage matrix has been completely replaced by proliferating fibroblast-like cells which can be traced along a continuous line of cells to the former perichondrium above and to the cell layer which replaced the articular cap cartilage. Fibrocartilage cells released from their lacunae are in all forms of what appears to be a transformation into the fibroblast-like cells of the developing ligament. In the caudal half of the symphysis the fibrocartilage matrix is in the final stage of dissolution. The interpubic separation was 1.5 mm. ×100.

FIG. 19. 6-week-old ♂ treated with estradiol and relaxin. The hormones had no effect, for the symphysis is the same as the untreated control male. ×100.

FIG. 20. 1-week-old ♀ control. The sparse glands are merely short, straight invaginations of the mucosal lining. ×53.

FIG. 21. 2-week-old ♀ treated with estradiol. The mucosa of the collapsed uterus is thrown into many folds. Only the distal portions of the glands are intact. ×53.

FIG. 22. 4-week-old ♀ control. The tortuous distal portions of the glands extend to the circular muscle layer. ×53.

FIG. 23. 4-week-old ♀ treated with estradiol. The serosa as well as the mucosa of the collapsed uterus is thrown into many folds. ×53.

FIG. 24. 6-week-old ♀ control.

FIG. 25. 6-week-old ♀ treated with estradiol. The folding of the mucosa of the collapsed uterus is marked and its reflections extend to the circular muscle layer. Thus the submucosa is found chiefly between the reflections of mucosa projecting into the lumen. ×53.

tion was twice the size of the controls of this group; however, the mass of the tissue had increased only slightly. The muscular wall of a collapsed uterus was markedly thinned, its lumen large, only the distal portions of a few glands present, and its mucosa greatly folded (Fig. 21). Mitoses were frequent in the epithelium and submucosa. When the collapsed uterus was distended, the uterine epithelium was presumably so stretched that the cells became low cuboidal and the mucosal surface completely smooth. The ducts of the glands contributed to the mucosal surface which lined the uterine lumen except for the distal-most portion of a few glands that remained embedded in a thin submucosa. The thin musculature was possibly only a few cells thick. When the fluid pressure was released by incising the uterus the contractility of the musculature allowed this tissue to approach somewhat its original size and shape, causing the mucosa, submucosa and serosa to be thrown into many folds.

Group III (*3 wks. E 5 ♀, 5 ♂*) The injected estrogen did not detectably affect the interpubic tissue of either sex.

The uteri were larger but the response to the injected estrogen was the same as that in the 2-week-old females.

Group IV (*4 wks. E 5 ♀, 5 ♂*) The symphyses of 3 females and 4 males resembled those of the untreated mice. In 2 females and 1 male the symphyses were flexible. Grossly they appeared swollen and histologically they differed from the controls in that the fibrocartilage lamina was wider. The lacunae were more widely separated than those of the controls and the fibrocartilage cells were spherical instead of flat. The matrix contained large bundles of collagen fibers. The swelling of the matrix probably caused the collagen bundles to become slightly separated and therefore visible with the staining method used. Although there undoubtedly was an increase in the transverse width of the interpubic tissue, it could not be considered significant for the interpubic separation was less than 0.5 mm.

The uteri responded to the injected estrogen in the same manner as those of the 2- and 3-week-old females (Fig. 23).

Group V (*5 wks. E 5 ♀, 5 ♂*) None of the males' symphyses showed a response to the injected estradiol. All of the females' symphyses were grossly swollen and flexible. Histologically the changes were the same as those found in the 4-week-old female symphyses which responded to the injected estrogen, except that in 3 females significant interpubic separations occurred, measuring 0.5, 0.5, and 0.8 mm. Also, a small amount of bone was resorbed from the cranio-dorsal surfaces of the medial ends of the pubes in these 3 females. A few osteoclasts were found along the surfaces being resorbed.

The uteri responded to the injected estrogen in the same manner as those of the 4-week-old females, except that the collapsed and contracted uteri did not show such a disparity in size when compared with the controls.

Group VI (*6 wks. E 5 ♀, 5 ♂*) None of the males' symphyses showed a detectable response to the injected estradiol. All of the females' symphyses were swollen and flexible. Histologically they were the same as the estradiol-responsive symphyses of the 5-week-old females. The symphyses of two females had an interpubic separation of 0.5 mm. and a small amount of bone was resorbed from the cranio-dorsal surfaces of the medial ends of the pubes. A few osteoclasts were found along the surfaces being resorbed.

The uteri responded to the injected estrogen in the same manner as those of the 5-week-old females. Following fixation the collapsed uteri were not much larger than those of the controls, however, their muscular walls were much thinner and their epithelium greatly folded (Fig. 25). As in all of the estrogen-injected mice of the other groups the uteri showed no pathological changes.

Estradiol- and Relaxin-treated Mice

Group I (*1 wk. E-R*[5] *5 ♀, 5 ♂*) The symphyses did not respond to the injected hormones; they were inflexible and structurally the same as the controls (Fig. 7). One female and 2 males had distended urinary bladders.

The uteri were distended and histologically comparable to those of the 1-week-old females which received estradiol alone. Within a small range of variation the uteri of the animals receiving estradiol and relaxin were similar to those of comparable mice which received estradiol alone. Thus, relaxin had no demonstrable effect on the uterus. Because of the similarity, a description of the uteri of the females which received both estradiol and relaxin will not be presented in the results of the subsequent groups.

Group II (*2 wks. E-R 5 ♀, 5 ♂*) The developing symphyses of all the males and 3 females showed no response to the injected hormones. Two females had flexible interpubic tissue; however, there was no significant increase in its transverse width. Histologically these symphyses, unlike the controls, had a slightly increased amount of matrix in the cranial portion of the fibrocartilage lamina. In this region the chondrocytes were more spherical than flattened. Also, small bundles of collagenous fibers in the matrix appeared slightly disrupted in their longitudinal courses.

One female and 2 males had distended urinary bladders.

Group III (*3 wks. E-R 5 ♀, 5 ♂*) All the females and 4 males had flexible symphyses. The only differences, when compared with the controls, were a swelling of the cells and matrix of the fibro-cartilage lamina and a slight disruption of its collagenous fibers. These changes were most pronounced in its cranio-dorsal portion. One female and 1 male each had a 0.5 mm. interpubic separation and grossly swollen symphyses.

Group IV (*4 wks. E-R 6 ♀, 6 ♂*) All of the symphyses were grossly swollen and flexible. Three males each had an interpubic separation of

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[5] Estrogen- and relaxin-treated.

0.5 mm. Three females had interpubic separations of 0.6, 0.6 and 0.7 mm. The histological appearance of the symphyses, including those of the males, was similar to that of the 4-week-old mice which responded to estrogen alone except that the fibrocartilage was more swollen, resulting in a wider lamina. Many lacunae in the cranial portion of the lamina contained 2 chondrocytes. The transitional zone between the fibrocartilage lamina and the articular caps was ill-defined (Figs. 14, 15).

Three females had interpubic separations of 0.8, 0.9, and 1.0 mm. Dorsally, the fibrocartilage matrix of the cranial third of their symphyses was probably undergoing dissolution for it showed no organized structures and stained lightly. Spherical chondrocytes in their lacunae were widely and irregularly scattered. These cells were in groups of 2 and 3, indicating that they were proliferating, presumably by amitotic division, for only a few cells undergoing mitoses were found. Fine bundles of long collagen fibers twisted in an irregular course between these lacunae. The fibrocartilage matrix of the caudal two-thirds of the symphyses was swollen and the cells within irregularly-spaced lacunae were spherical (Fig. 16). The articular caps were absent in the cranial third, and thin in the caudal two-thirds of these symphyses. A thick compact layer of proliferating fibroblast-like cells lined the pubic bones where the articular cartilage was absent. This layer was directly continuous with the remaining articular cartilage through a short zone of transition from fibroblast-like cells to hyaline cartilage. No degenerating cells were found in the thin articular caps of the animals in the preceding or subsequent groups.

The symphyses all appeared swollen except those of the 3 females with the greatest interpubic separations. *In situ*, especially under tension, their interpubic tissue was a flattened translucent band.

Group V (5 wks. E-R 5 ♀, 5 ♂) The pubic symphysis of each mouse was flexible. When the interpubic separations were 0.7 mm. or less, the interpubic tissue appeared swollen; with separations greater than 0.7 mm., it appeared as a flattened translucent band. The males' interpubic separations averaged 0.6 mm. The range was 0.5–0.9 mm. In the females the average separation was 1.2 mm. The range was 0.8–2.0 mm. In the mice with interpubic separations of 0.8 mm. or less (1 ♀, 4 ♂) the interpubic tissue resembled those of the 4-week-old estradiol- and relaxin-injected mice that had separations of 0.7 mm. or less. The male with the widest separation (0.9 mm.) had interpubic tissue similar to those of the 4-week-old estradiol- and relaxin-injected females with separations of 0.8 mm. or more. The caudal two-thirds of the fibrocartilage, however, was not as swollen as it was in the females. The chondrocytes were compact and oval (Fig. 17).

In the 4 females with a separation of 1.0 mm. or more the fibrocartilage matrix was almost completely resorbed (Fig. 18). The process probably

occurred first in the cranial portion of the fibrocartilage in the same manner described for the 4-week-old estradiol- and relaxin-injected females which had separations of 0.8 mm. or more. All of the matrix was absent in the cranial half of the symphysis. The fibrocartilage cells, freed from their lacunae, appeared to be in all stages of transformation into fibroblast-like cells by elongating and orienting themselves in a transverse direction.

Many small bundles of collagen fibers extended somewhat transversely among what appeared to be elongating fibrocartilage cells. In the caudal half of the symphysis the matrix was nearly all resorbed. Widely separated small bundles of collagenous fibers twisted in an irregular course in this area. The cells in this region were also widely separated. In the midline some "floating" cells in groups of 2 and 3 were spherical and still appeared more like fibrocartilage cells than fibroblasts. Cell division was presumed to be chiefly amitotic for very few cells were found undergoing mitosis. The articular caps were lacking in the cranial portion of the symphysis and very thin in the caudal part. A dense layer of proliferating fibroblast-like cells formed a continuous lining along the medial surface of the remaining articular caps and the pubic bones where the caps were lacking. Many fibroblast-like cells formed a continuous line running in a cranio-medial direction from the dense layer lining the remaining articular caps to the areas where the fibrocartilage matrix had disappeared or was in the process of doing so.

A small amount of bone was resorbed from the cranio-dorsal surfaces of the medial ends of the pubes of the females. A few osteoclasts were found along the surfaces being resorbed.

Three of the males had distended urinary bladders. One female had an undistended uterus which was about the same in size and structure as the control. This female had an interpubic separation of 1.0 mm.

Group VI (6 wks. E-R 5 ♀, 5 ♂) Only 1 male had a 0.5 mm. interpubic separation. Histologically the symphysial response was slight, for the only evident change was a swollen fibrocartilage matrix. The other males showed no response. Their symphyses were inflexible and structurally identical to the controls (Fig. 19). All of the females' symphyses showed a definite response. The symphysis of 1 female, with a separation of 0.7 mm., resembled that of the 5-week-old estradiol- and relaxin-injected female which had an 0.8 mm. separation. Four females, with separations of 1.0, 1.2, 1.2, and 1.3 mm., were similar to the estradiol- and relaxin-injected 5-week-old females with separations of 1.0 mm. or more. Some bone was resorbed from the cranio-dorsal surfaces of the medial ends of the pubes in three of the 4 females. A few osteoclasts were found along the surfaces being resorbed.

One female with a 1.2 mm. interpubic separation had an undistended uterus.

DISCUSSION

The changes occurring in the normal mouse symphysial tissue during the first 6 weeks of life are primarily those of differentiation, for only a small increase in over-all size occurred. The formation of the symphysis in mice as a secondary development from an original bar of hyaline cartilage is similar to that which Ruth (1932) described in a study of the rat, rabbit, cat, armadillo, pig, prairie dog and man.

At the end of the 4th postnatal week there was a relatively wide symphysial fibrocartilage lamina and thin articular hyaline cartilage caps. From this period on, the well-ossified pubic bones at the symphysis were closely approximated. The fibrocartilage cells and their lacunae became extremely flattened and the bulk of the surrounding matrix greatly reduced. These changes could have been the result of an increased polymerization of the matrix ground substance. It is interesting to note that as forces within the developing interpubic tissue appeared to draw the pubic bones together at the symphysis, the tension of the bulk of the laterally-attached pelvic musculature tended to separate them. The laterally directed muscle tension must have been considerable in mice 3 to 6 weeks of age for they are extremely active.

Because the amount of hormone injected was the same throughout the experiment, the developing symphysial tissue of mice 1 to 3 weeks of age displayed an extreme lack of sensitivity, for they received a high dose per body weight. The average weight of the 1-week-old mice was 4 gm; at the end of 6 weeks it was 20 gm.

The response of the young estradiol- and relaxin-treated mice of the present study indicated that the symphysial tissue of many of the estradiol-treated mice of a comparable age which showed no response probably would have produced a detectable response if relaxin had also been in-injected.

In the present experiment none of the treated males responded as well as the females of a comparable age. This is true when gonadectomized adult male and female mice are treated with the same hormones (Crelin, 1954a). The androgen secreted by the testes may have been the chief factor in the inhibition of response in the immature males. The sudden lack of response of the 6-week-old males to the injected estradiol and relaxin may have been due to either a sudden increase in androgen production by the testes; or the testicular androgen output, which had been gradually increasing, reached a level sufficient to prevent the injected estrogen from affecting the symphysial tissue. The symphysial tissue of the adult castrated male responds to identical hormonal treatment (unpublished data).

Following estrogen treatment in the guinea pig the water content of the symphysial tissue is increased (Talmage, 1950). The ground substance of the estrogen-primed symphysial tissue of the guinea pig is believed to be

depolymerized following relaxin treatment. The normally high state of intermolecular organization of the ground substance becomes more loosely arranged, causing the tissue to assume a more fluid consistency and a degree of mobility (Perl and Catchpole, 1950). A recent study by Hall (1954) indicates that the explanation presented for the changes occurring in the guinea pig may be applicable to those occurring in the mouse.

Perl and Catchpole (1950) did not study the collagenous fibers in the guinea pig but believed that if their concept of the fibers being secured in a matrix of ground substance is true, a weakening of the ground substance should lead to disorientation of the fibers, irrespective of intrinsic changes in the fibers themselves. Such a disorientation of the fibers occurred in the treated mice of the present study. However, the fate of the collagenous fibers could not be adequately determined because of the staining method used.

The pattern of changes of the immature symphysial tissue in response to the injected hormones was consistent with that which occurs during normal pregnancy in the mouse. The swelling of the cartilage, its breakdown and replacement by collagenous connective tissue began in the cranio-dorsal region and then extended caudally. Because of the many symphyses which were in different stages of the consistent step-by-step pattern of cartilage breakdown and ligament replacement, it was possible to construct from these static stages a continuous process in order to speculate as to the origin of the fibroblast-like cells which formed the ligament. In the earliest stages the fibroblast-like cells are probably derived from (a) chondrocytes released from their lacunae in the cranial region of the articular caps opposite that portion of the fibrocartilage lamina where its matrix first undergoes dissolution, (b) fibrocartilage cells which are released from their lucunae as the matrix breaks down, (c) fibroblasts from the cranial, cranio-dorsal and cranio-ventral portions of the symphysial perichondrium. As the process proceeds caudally the released fibrocartilage cells and adjacent articular hyaline cartilage cells, as well as cells from the adjacent perichondrium, probably all contribute to ligament formation. One of the final contributing areas is the caudal portion of the perichondrium. Hall (1954), in a study of interpubic ligament formation in the pregnant mouse, states that the final source of cells which contribute to ligament formation is probably released osteocytes or cells of the marrow which become fibroblast-like cells and migrate into the area following sufficient resorption of the medial ends of the pubic bones.

The distension of the uterus in response to estradiol is presumably due to glandular secretion and serous exudate which is retained within the lumen by the sphincter action of the cervical musculature. This distension, though more pronounced, was similar to that which normally occurs during proestrus in the mouse.

The distended urinary bladders in the mice from 1 to 3 weeks of age is

consistent with previous studies in that large amounts of estrogen per body weight are necessary to produce this effect within a relatively short period of time. The distension was due to a constriction of the urethra in the region of the pubic symphysis, which is the same area of constriction described by Gardner (1935) in male mice following injections of large amounts of estrogen.

SUMMARY

Newborn male and female mice were arranged into 3 groups: (1) controls, (2) estrogen-treated, (3) estrogen- and relaxin-treated. Animals from each group were sacrificed at weekly intervals from the end of the 1st to the end of the 6th postnatal week. Hormonal treatment began 5 days before necropsy. Estrogen-treated mice received 1 μg. estradiol benzoate subcutaneously on the 1st and 3rd day. The estrogen- and relaxin-treated mice received the same estradiol treatment plus a subcutaneous injection of 100 G. P. units of relaxin 24 hours before necropsy.

At 1 week the ventral portion of the control pelves consisted entirely of hyaline cartilage. At 3 weeks an immature pubic symphysis was present. By 4 weeks the pubes completely formed the lateral walls of the symphysis and the symphysial fibrocartilage cells were compact and flattened. At 6 weeks of age the bony pelves were less than one-half larger in size than at 1 week and showed a sexual dimorphism. These symphyses resembled the adult type.

The males' symphyses showed no response to estradiol treatment, whereas the interpubic cartilage of the 4- to 6-week-old females were swollen and flexible. In the estradiol- and relaxin-treated males, flexible and swollen interpubic cartilage occurred at 3 weeks and increased in degree up to 5 weeks. At 6 weeks all of the males' symphyses, except one, failed to respond to estrogen and relaxin treatment. Swollen and flexible interpubic cartilage occurred by 2 weeks in the estradiol- and relaxin-treated females and increased in degree up to 4 weeks, when the matrix in the cranial portion had undergone dissolution. In nearly all of the 5- and 6-week-old females treated with estradiol and relaxin the matrix of the interpubic cartilage was almost completely resorbed with ligament formation occurring in the cranial half. Interpubic separations up to 2.0 mm. occurred in these females. The failure of the 6-week-old males to respond to hormonal treatment, the underlying causes of the changes in the interpubic cartilage, and the source of cells for ligament formation are discussed.

At 1 week the uterine glands of the control female mice were sparsely distributed; consisting of short, straight invaginations of the lumen epithelium. By 3 weeks the distal ends of the glands had begun to coil. At 4 weeks the distal ends extended to the circular muscle layer and were quite tortuous. The uteri of the 6-week-old control females were 4 times larger in

diameter than those of the 1-week-old controls. All of the hormonally-treated females, except two, had uteri distended with fluid.

REFERENCES

ALLEN, E.: *Amer. J. Anat.* **31**: 439. 1923.

BURROWS, H.: *Biological Actions of Sex Hormones.* N. Y. Cambridge University Press. 1949.

CRELIN, E. S.: *Amer. J. Anat.* **95**: 47. 1954a.

CRELIN, E. S.: *Anat. Rec.* **120**: 23. 1954b.

CRELIN, E. S.: *Proc. Soc. Exper. Biol. & Med.* **86**: 22. 1954c.

CRELIN, E. S. AND A. L. HAINES: *Endocrinology* **56**: 461. 1955.

DORFMAN, R. I., R. W. MARSTERS AND J. DINERSTEIN.: *Endocrinology* **52**: 204. 1953.

GARDNER, W. U.: *Amer. J. Anat.* **59**: 459. 1936.

GARDNER, W. U.: *Proc. Soc. Exper. Biol. & Med.* **33**: 104. 1935.

HALL, K.: *J. Endocrinol.* **5**: 174. 1947.

HALL, K.: *J. Endocrinol.* **5**: 314. 1948.

HALL, K.: *J. Endocrinol.* **7**: 54. 1950.

HALL, K.: *J. Endocrinol.* **11**: 210. 1954.

KLIMAN, B., H. A. SALHANIK, E. H. FRIEDEN AND F. L. HISAW: *Endocrinology* **53**: 403. 1943a.

KLIMAN, B., H. A. SALHANIK AND M. X. ZARROW: *Endocrinology* **53**: 391. 1953b.

KLIMAN, B. AND H. A. SALHANIK: *Proc. Soc. Exper. Biol. & Med.* **81**: 201. 1952.

PERL, E. AND H. R. CATCHPOLE: *Arch. of Path.* **50**: 233. 1950.

RUTH, E. B.: *Anat. Rec.* **53**: 207. 1932.

TALMAGE, R. V.: *Endocrinology* **47**: 75. 1950.

TRENTIN, J. J.: *Proc. Soc. Exper. Biol. & Med.* **78**: 9. 1951.

STUDIES ON THE ROLE OF GROWTH HORMONE IN PREGNANCY[1]

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IT HAS been postulated by various workers that growth hormone might play a part in gestation in view of the rapid fetal growth and marked nitrogen retention. Attempts to detect this hormone in the urine of pregnant women and blood of pregnant goats have met with failure. (Cotes and Young, 1951.) Results obtained in our laboratory (G. Beaton *et al.*, 1954) have shown that there is a sudden increase in fetal growth and a simultaneous appearance of certain biochemical changes at the 15th day of pregnancy in the rat. These changes include haemodilution, lowered rate of urea formation by liver slices, lowered liver alanine-glutamic transaminase activity, increased liver weight and changes in carcass composition. The sudden simultaneous appearance of these alterations suggested the action of a hormone factor. A previous study (G. Beaton *et al.*, 1953), in which purified growth hormone was administered to normal rats indicated similar enzymic changes. Further experiments were undertaken to investigate the apparent role of growth hormone in pregnancy in the rat.

METHODS

In the first three experiments female Wistar-strain rats of the Connaught Laboratories stock were employed. The initial average body weights of the animals in these experiments were 250 gm., 268 gm. and 240 gm.; all experimental groups within each study were balanced at the beginning of the experiment. In these three studies the animals were placed in individual screen-bottomed cages after mating, and were provided *ad libitum*, with a 20% casein, 20% corn oil diet adequate for normal growth of young rats (J. Beaton *et al.*, 1953). The animals were weighed three times weekly. At the completion of the experiments the animals were fasted for 18–20 hours and then killed by stunning and decapitation. Blood was collected from the neck in heparinized tubes and livers were removed immediately and prepared for the enzyme assays.

In the fourth experiment, both hypophysectomized and unoperated Sprague-Dawley Strain rats were obtained from Hormone Assays Corporation, Chicago. The operation was carried out in the morning of the 12th day of gestation and on the same day, after arrival in our laboratory, the animals were placed in individual cages and provided *ad libitum* with ground prepared fox chow[2] and water. The animals were weighed

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[2] Master Fox Breeder Ration, Toronto Elevators Limited: crude protein, 20%; crude fat, 3.5%; crude fiber, 5%.

daily and were killed on the 19th day of gestation following an 18-hour fast.

Hormone preparations employed in these studies were anterior pituitary growth hormone (Connaught Laboratories, lot 200), beef anterior pituitary powder (courtesy of Armour Laboratories, lot 305), equine estrogenic substances (Ayerst, McKenna and Harrison) and progesterone (Nutritional Biochemical Corp.). The estrogenic substances and progesterone were dissolved in sesame oil (Ciba Co.).

Alanine-glutamic transaminase activity was determined by the procedure of Caldwell and McHenry (1953); results are expressed as μm. pyruvate-CO_2 formed per mg. wet tissue per hour. The d-amino acid oxidase activity of liver homogenates was determined by a modified procedure of Lan (1943). In a Warburg flask were placed 0.4 ml. of a 20% aqueous homogenate and 1.5 ml. of pyrophosphate buffer at pH 8.3; 0.5 ml. of 0.6 *M* DL-alanine was placed in a side arm and 0.2 ml. of 5% potassium hydroxide and a filter paper wick were placed in the center well. Pure oxygen was used as the gas phase. Following a 10-minute equilibration period at 37° C. the system was closed and after a further 5 minutes, the initial reading was taken, the vessels tipped and readings taken every 10 minutes. The oxygen uptake was found to be linear for approximately one hour. Results are expressed as μl. oxygen taken up per hour per mg. wet tissue.

Blood assays were performed by the following procedures: urea (Archibald, 1945), amino nitrogen (Frame *et al.*, 1943), packed cell volume by the standard procedure. Statistical analysis was carried out employing the "t" test.

RESULTS

Experiment I. Supplemental Growth Hormone

One group of pregnant rats and three groups of non-pregnant animals were included in this experiment. Daily intraperitoneal injections of 2 mg. growth hormone in 0.5 ml. of alkaline saline were given to one group of non-pregnant rats. Another group received daily injections of 0.5 ml. of alkaline saline. These injections were carried on for 19 days including the day of killing. The average gestation time was 19.5 days. The results are shown in Table 1.

As has been seen in previous studies (G. Beaton *et al.*, 1954) there was an increased liver weight and decreased packed cell volume, blood amino nitrogen, and liver alanine-glutamic transaminase activity. A significant depression of d-amino acid oxidase activity was also found in the present study (pregnant = 0.24, control = 0.92, difference significant at the 1% level). The administration of growth hormone was found to bring about many similar changes. Body weight and liver weight were increased and alanine-glutamic transaminase was decreased. As in the pregnant animals, there was also a lowered d-amino acid oxidase activity in growth hormone-treated rats (growth hormone = 0.75, saline = 0.94, difference significant at the 5% level). Growth hormone, however, had no effect on the packed cell volume and actually brought about an increase in blood amino nitrogen. The changes induced by the hormone treatment were not as great as those seen during gestation, even though they were, in many cases, in the same direction. Previous studies, not reported here, have shown similar findings following growth hormone treatment.

TABLE I. METABOLIC CHANGES IN PREGNANCY AND AFTER GROWTH HORMONE TREATMENT

	No. of rats	Body weight gain, gm.	Liver weight as % body wt.	Packed cell volume, %	Blood urea, mg. %	Blood amino N, mg. %	Trans-aminase
Experiment I							
Non-pregnant	6	43	2.5	54	34	8.9	41
N.P.+Saline	4	48	2.5	49	35	8.4	48
Pregnant	4	128	2.8	46	34	7.9	15
N.P.+G.H.	5	76	2.8	53	34	9.7	34
Experiment II							
Control	8	15	2.6	53	32	9.0	47
S.H.	8	26	2.8	51	33	9.0	44
G.H.	8	31	2.8	49	29	11.2	26
S.H.+G.H.	8	47	3.0	46	28	11.1	20
Experiment III							
Control	8	6	2.7	49	31	8.0	31
Pituitary powder (6 mg.)	8	44	2.8	50	30	8.5	30
Experiment IV							
Pregnant Control	8	57	3.0	42	41	9.2	30
Hyp'd pregnant	2	82	2.3	36	44	7.8	11
Hyp'd pregnant+G.H.	5	82	2.3	38	35	8.2	10
Control	11	26	2.6	54	32	11.0	60
Hyp'd control	13	−7	2.1	57	68	10.7	54
Hyp'd control+G.H.	9	11	2.0	53	40	10.1	16

Experiment II. Supplemental Sex and Growth Hormones

Thirty-two animals were divided into 4 groups comparable with respect to number and initial average body weight and given the following daily subcutaneous injections.

Group	*Injections*
Control	0.2 ml. sesame oil, 0.5 ml. saline
S.H.	0.2 ml. sex hormone,[3] 0.5 ml. saline
G.H.	0.2 ml. sesame oil, 0.5 ml. growth hormone[4]
S.H., G.H.	0.2 ml. sex hormone,[3] 0.5 ml. growth hormone[3]

The animals in groups S.H. and S.H., G.H. received daily subcutaneous injections of 1.5 μg. estrogen for 2 days before the other hormone injections were started. The dosages of sex hormones used in this experiment were similar to those found by Lyons (1943) to be capable of supporting gestation in hypophysectomized-oophorectomized rats. The same dosages prevented abortion in vitamin B_6-deficient and protein-deprived rats (Nelson *et al.*, 1951; Nelson and Evans, 1954). The daily injections were carried on for 14 days. The experimental results are shown in Table 1.

The body weight gains induced by growth hormone (G. H.), sex hormones (S.H.) or both when administered to these non-pregnant rats can be explained by the increased food intakes shown by these groups (food

[3] "Sex hormone"—1.5 μg. estrogen+3 mg. progesterone per 0.2 ml. sesame oil.

[4] Growth hormone—2 mg. growth hormone per 0.5 ml. saline.

intake in gm./rat/day: control, 12.5; S.H., 14.4; G.H., 14.4; S.H., G.H., 15.0). The administration of these hormones had a very real effect on uterus weight; in both the control and growth hormone-treated groups, the average weight was 0.76 gm., whereas in the two groups receiving the sex hormones the average weight was 0.48 gm., the difference being significant at the 1% level. This effect on uterus weight confirms the potency of these hormones.

The effects of growth hormone administration were similar to those seen in the previous study. Growth hormone produces some but not all of the changes seen during pregnancy. The sex hormone administration per se produced only slight changes. However, when the two preparations were administered together, marked changes were produced in body weight gain, liver weight, and alanine-glutamic transaminase activity. This joint treatment also induced a fall in the packed cell volume comparable with that seen during gestation. Blood amino nitrogen was increased by the treatment, rather than lowered as in pregnancy. It would appear, then, that growth hormone in the presence of the sex hormones, which are believed to be definitely available during gestation, can produce many of the biological changes typical of pregnancy.

Experiment III. Supplemental Pituitary Powder

In order to test the possible action of other pituitary hormones, it was decided to administer anterior pituitary powder in a similar manner to that employed with growth hormone. Twenty-four animals were divided into three groups, comparable with respect to number and initial average body weight. The animals of one group received daily subcutaneous injections of 0.75 ml. of 0.9% saline; those of the second and third groups received daily subcutaneous injections of 0.5 and 1.0 ml. of pituitary powder solution respectively (6 mg. per ml.). The injections were carried out for 19 days. Assays were carried out as before. The treated groups ate more food (average food intake in gm./rat/day: control, 14.2; 3 mg., 16.7; 6 mg., 16.3) and gained more weight than did the controls. Liver weight increased in the treated groups, but only in proportion to body weight. No other differences were observed between groups; since little alteration occurred as the dosage was increased from 3 to 6 mg./rat/day, only the latter results are shown in Table 1.

Even though this experiment failed to show any effects of anterior pituitary powder administration other than the above mentioned, it did suggest that the results obtained in the pregnancy and growth hormone studies cannot be explained simply on the basis of an increased food intake.

Experiment IV. Hypophysectomy and Hormone Supplements

Campbell *et al.* (1953), reported that, if pregnant rats were hypophysectomized on the thirteenth to fifteenth day of gestation, pregnancy was

uninterrupted. Fetal and maternal weight gains were unaffected. Much earlier, Pencharz and Long (1933) reported the effects of hypophysectomy of rats at various stages of gestation. They found that, if the operation was performed before the eleventh day, resorption followed; if after the eleventh day, pregnancy was maintained. It was decided to repeat these experiments and study the biochemical alterations.

Rats were hypophysectomized (by Hormone Assays Corporation) on the twelfth day of gestation; operated and unoperated animals were divided into groups as shown in Table 1. The animals were given injections of saline or growth hormone as indicated for a period of six days, and were killed in the fasted state on the seventh day. Experimental results are shown in Table 1. Hypophysectomy, with or without growth hormone, had no effect on litter size or fetal weight.

From the results shown in Table 1 it can be seen that hypophysectomy of pregnant rats led to a decrease in liver weight, alanine-glutamic transaminase, packed cell volume and blood amino nitrogen. None of these differences was affected by the administration of growth hormone. Hypophysectomy of non-pregnant rats led to a marked fall in liver weight, a rise in packed cell volume and a great rise in blood urea. Growth hormone returned the packed cell volume to the normal level and largely restored the blood urea to the non-pregnant level. A marked fall in alanine-glutamic transaminase resulted from the administration of growth hormone, in agreement with the findings in intact rats.

Although food intakes were not followed accurately, estimates indicated that hypophysectomy of non-pregnant animals led to a drastic decrease; this was evident in pregnant animals also, but was not as marked. Growth hormone administration seemed to have little effect on the food intake.

DISCUSSION

Statistical treatment of the experimental data, using the "t" test, has shown that the following observations are significant at either the 1% or 2% level of probability:

Experiment I

Pregnant vs. non-pregnant: body weight gain, liver weight, P.C.V., alanine-glutamic transaminase, d-amino acid oxidase.

G.H. vs. saline (non-pregnant): alanine-glutamic transaminase.

Experiment II

Control vs. S.H.: liver weight.

Control vs. G.H.: body weight gain, liver weight, alanine-glutamic transaminase.

Control vs. S.H. and G.H.: body weight gain, liver weight, alanine-glutamic transaminase, blood amino nitrogen.

Experiment III

Control vs. pituitary powder: body weight gain.

Experiment IV

Pregnant control vs. hypophysectomized pregnant: liver weight, alanine-glutamic transaminase, P.C.V., blood amino nitrogen.

Hypophysectomized pregnant vs. hypophysectomized control: liver weight, alanine-glutamic transaminase, P.C.V., blood urea, blood amino nitrogen.

Hypophysectomized control vs. control: liver weight, P.C.V., blood urea.

Hypophysectomized control + G.H. vs. hypophysectomized control: alanine-glutamic transaminase, blood urea.

It has been shown that many of the metabolic changes characteristic of pregnancy in the rat can be produced in the non-pregnant animal by the administration of growth hormone, especially in the presence of estrogen and progesterone. These effects were not produced by the administration of anterior pituitary powder, although there was an increase in food intake and body weight. Blood amino nitrogen was elevated by growth hormone administration and lowered by pregnancy. It is possible that, in the pregnant animal, amino acids are transported across the placenta and concentrated in the fetal circulation, to be used for the synthesis of fetal protein. At birth, there is a much higher concentration of amino nitrogen in the fetal circulation than in the maternal blood (Clemenston, 1954). Growth hormone seems to stimulate the production of amino acids and in the absence of the rapidly growting fetus, the blood level rises rather than falls.

Hypophysectomy did not interrupt the course of gestation. Biochemically it accentuated the changes of a normal pregnancy. This may be a reflection of the adaptation of the rat to a double stress on protein metabolism. It is interesting to note that hypophysectomy of non-pregnant rats led to a marked increase in blood urea; however, this was not seen if growth hormone was given or if the animal was pregnant. From the results of this last experiment it might appear that growth hormone had no function in the latter part of gestation; however, it should be remembered that previous studies involved the addition of growth hormone in the presence of the other normal pituitary hormones, whereas the present experiment involves the complete removal of all the pituitary hormones and the re-addition of growth hormone only. There is also the possibility that the fetal pituitaries produce sufficient growth hormone following maternal hypophysectomy to meet the requirements of the pregnant animal. Other workers have shown that the fetal adrenals hypertrophy following maternal adrenalectomy in rats (Knobil and Briggs, 1954) and such is believed to occur with the fetal pancreas in diabetics. Thus, the last experi-

ment does not invalidate the concept of a role of growth hormone; indeed, the effect of pregnancy on the blood urea of hypophysectomized rats is suggestive of the action of growth hormone. Other evidence presented is suggestive of a role of growth hormone in gestation. Until a suitable procedure is developed for the measurement of growth hormone in blood, no definite conclusions can be drawn regarding the importance of this hormone in normal pregnancy.

SUMMARY

Administration of growth hormone to non-pregnant female rats caused significant decreases in hepatic alanine-glutamic transaminase and in d-amino acid oxidase, resembling alterations observed in pregnant animals. The hormone treatment caused less than significant changes in body weight, liver weight and packed cell volume but the trends were similar to those of pregnancy. Resemblance of biochemical changes to those characteristic of pregnancy was enhanced by simultaneous administration of estrogen, progesterone and growth hormone. Crude pituitary powder was not effective. Hypophysectomy on the 12th day of gestation did not terminate pregnancy but did alter some of the biochemical effects of pregnancy (packed cell volume, blood urea, alanine-glutamic transaminase). It is suggested that growth hormone may be responsible for at least some metabolic alterations during pregnancy in the rat.

REFERENCES

ARCHIBALD, R. M.: *J. Biol. Chem.* **157:** 507. 1945.

BEATON, G. H., G. OZAWA, J. R. BEATON AND E. W. MCHENRY: *Proc. Soc. Exper. Biol. & Med.* **83:** 781. 1953.

BEATON, G. H., J. BEARE, M. H. RYU AND E. W. MCHENRY: *J. Nutrit.* **54:** 291. 1954.

BEATON, J. R., J. L. BEARE, J. M. WHITE AND E. W. MCHENRY: *J. Biol. Chem.*, **200:** 715. 1953.

CALDWELL, E. F. AND E. W. MCHENRY: *Arch. Biochem. & Biophys.* **45:** 97. 1953.

CAMPBELL, R. M., I. R. INNES AND H. W. KOSTERLITZ: *J. Endocrinol.* **9:** 68. 1953.

CLEMENSTON, C. A. B.: *J. Obstet. Gynecol. Brit. Empire* **61:** 364. 1954.

COTES, P. M. AND F. G. YOUNG: *Biochem. J.* **49:** lix. 1951.

FRAME, E. G., J. A. RUSSELL AND A. E. WILHELMI: *J. Biol. Chem.* **149:** 255. 1943.

KNOBIL, E. AND F. N. BRIGGS: *Fed. Proc.* **13:** 80. 1954.

LAN, T. H.: *J. Biol. Chem.* **151:** 171. 1943.

LYONS, W. R.: *Proc. Soc. Exper. Biol. & Med.* **54:** 65. 1943.

NELSON, M. M. AND H. M. EVANS: *Endocrinology* **55:** 543. 1954.

NELSON, M. M., W. R. LYONS AND H. M. EVANS: *Endocrinology* **48:** 726. 1951.

PENCHARZ, R. I. AND J. A. LONG: *Am. J. Anat.* **53:** 117. 1933.

NOTES AND COMMENTS

DEMONSTRATION OF THYROIDAL RESPONSE TO EXOGENOUS THYROTROPIN IN RATS WITH ANTERIOR HYPOTHALAMIC LESIONS

Midline hypothalamic lesions between the paraventricular nucleus and median eminence will prevent the usual goitrogenic response to propylthiouracil feeding in rats (Greer, 1951; Bogdanove and Halmi, 1953; Greer and Erwin, 1954). This has been interpreted as an interference with the normal control of thyrotropin secretion in such animals. It is possible, however, that damage to the hypothalamus might inhibit, in some as yet unknown manner, the response of the thyroid gland to thyrotopin. The secretion of thyrotropin might thus continue unimpaired but would not be detected because of failure of the end organ response.

The present investigation was designed to study this possibility.

MATERIALS AND METHODS

Young adult female Holtzman rats of the same age were used. Bilateral anterior hypothalamic lesions were made in Groups I, II and III by a method previously described (Greer, 1951). The lesions were 6.0 mm. anterior to the ear bars, 1.0 mm. from the base of the skull, and 0.5 mm. lateral to the midline. A direct current of 6 ma for 15 seconds was used. Lesions in this area have been found to prevent the goitrogenic response to propylthiouracil feeding.

One week postoperatively Groups II, III and V were fed 0.15% propylthiouracil in ground Purina chow. Groups I and IV were fed Purina chow only. The animals were killed after 10 days on this diet. Group III in addition received 20 mg. Parke, Davis Thyrotropin Preparation (lot 50P4) dissolved in physiologic saline by subcutaneous injection once daily on days 3 through 8 of the 10 day experimental period.

The thyroid glands were dissected cleanly and weighed on a 200 mg. Roller-Smith torsion balance.

RESULTS AND DISCUSSION

The results are given in Table 1. It can be seen that the hypothalamic lesions com pletely prevented the goitrogenic response to propylthiouracil feeding seen in the control animals. The thyroid glands of the animals with hypothalamic lesions were capable of responding to exogenous thyrotropin, however, with a doubling of their weight. This response is roughly equivalent to that seen in the intact control rats receiving propylthiouracil, which would be expected to have a maximal secretion of endogeneous thyrotropin. This indicates that failure of the goitrogenic response to propylthiouracil in rats with anterior hypothalamic lesions respresents an inability of the pituitary of these animals sufficiently to augment its secretion of thyrotropin.

TABLE 1. EFFECT OF TSH ON THYROID WEIGHT OF RATS WITH HYPOTHALAMIC LESIONS

Group	No. Animals	Body Weight—gm.		Thyroid mg.	Hypothalamic Lesion	Propylthiouracil	TSH
		Initial	Final				
I	6	233.5±1.3*	298.7± 9.1	15.4±1.0	+	0	0
II	6	219 ±1.4	222 ± 6	14.7±1.1	+	+	0
III	6	236.5±1.3	224.7±16.7	30.8±1.8	+	+	+
IV	6	231.2±1.4	246.4± 1.6	16.4±1.1	0	0	0
V	6	228.7±1.2	214.6± 3.3	38.2±1.8	0	+	0

* Standard error of mean.

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Although there is no direct proof that the rats which received exogenous thyrotropin would not have had a goitrogenic response to propylthiouracil feeding, the control animals with lesions uniformly failed to exhibit thyroid hypertrophy in response to this treatment. Previous investigations with several hundred rats have also indicated that destruction of this area will inhibit the normal response to goitrogens (Greer and Erwin, 1954). The animals receiving thyrotropin were given propylthiouracil in addition because of the reported potentiation of the former by the latter (Halmi and Spirtos, 1954).

SUMMARY

Anterior hypothalamic lesions in the rat do not interfere with the response of the thyroid gland to thyrotropin. The failure of a goitrogenic response to propylthiouracil administration in such animals therefore probably represents an inability of the pituitary sufficiently to augment its secretion of thyrotropin.

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REFERENCES

GREER, M. A.: *Proc. Soc. Exper. Biol. & Med.* **77**: 603. 1951.
BOGDANOVE, E. M. AND N. S. HALMI: *Endocrinology* **53**: 274. 1953.
GREER, M. A. AND H. ERWIN: *J. Clin. Invest.* **33**: 938. 1954.
HALMI, N. S. AND B. N. SPIRTOS, *Endocrinology* **55**: 613. 1954.

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THE EFFECTS OF THE ACETIC ACID ANALOGS OF THYROXINE AND TRIIODOTHYRONINE ON GLYCOLYSIS IN ASCITES TUMOR CELLS *IN VITRO*

Thibault and Pitt-Rivers (1955) have reported that triiodothyroacetic acid (Triac) and tetraiodothyroacetic acid (Tetrac) show an immediate stimulatory effect on the metabolic rate of thyroidectomized rats and on the *in vitro* oxygen consumption of liver and kidney slices from these animals. In this communication a second dramatic effect of these acetic acid derivatives, namely, the acceleration of glycolysis of ascites tumor cells is discussed. Other compounds such as triiodothyronine, 3:5 diiodo-4-hydroxyphenylacetic acid (DIHPA), and 2,4-dinitrophenol (DNP) were also investigated and compared with Triac and Tetrac.

METHODS

Ehrlich mouse carcinoma ascites tumor cells were washed several times in 0.9% saline to remove blood cells and then resuspended and washed once in the buffer in which the manometric measurements were carried out. Sarcoma 37 cells were treated similarly.

Aerobic and anaerobic glycolysis was measured in the conventional Warburg apparatus in a Krebs-Henseleit bicarbonate buffer, pH 7.4. The atmosphere for the aerobic glycolysis was 95% O_2–5% CO_2, and for anaerobic glycolysis 95 %N_2–5% CO_2. Oxygen consumption was determined manometrically in a Krebs-Henseleit phosphate buffer, pH 7.4, in air. Ten to twenty micromoles of glucose in 0.9% saline and varying concentrations of hormone were placed in the side arm of the vessel. The final volume was made to 2.0 ml with the appropriate buffer. After ten minutes temperature equilibration at 37° a zero time reading was taken, and the contents of the side arm were tipped into the cell suspension.

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For chemical studies suitable aliquots were withdrawn and glucose disappearance determined by the Nelson method (1900) and lactic acid production according to the procedure of Barker and Summerson (1900).

RESULTS AND DISCUSSION

Stimulation of Aerobic Glycolysis by Triac and Tetrac—The effect of Triac on aerobic glycolysis of Ehrlich carcinoma tumor cells is shown in table 1. It should be noted that the stimulation occurs immediately after the Triac is tipped into the main compartment of the Warburg vessel and the rise is linear. In this experiment the maximum stimulation of 133%* occurred at a concentration of 50μg. Triac per ml. A stimulation of aerobic glycolysis has also been obtained with Sarcoma 37 cells from virus-susceptible and C_3H mice. (Table 1) The ratio of cell count to hormone concentration is an important factor in the degree of stimulation of aerobic glycolysis (Tables 1 and 2). Although a large series of experiments has not been carried out to determine the optimal concentration at various cell counts, it was necessary to work over a large range of concentrations in order to obtain these results with various types of cells.

Chemical balance studies have been carried out to establish the source of the extra CO_2 resulting from the addition of Triac and DNP to glycolyzing tumor suspensions. It has been determined that CO_2 and lactic acid measurements are essentially identical and equal to that expected from glucose disappearance.

Tetrac has been shown to be about 70% as effective as Triac in stimulation of aerobic glycolysis of Ehrlich carcinoma tumor cells (Table 2).

At almost the same cell concentration 50 μg. of Triac per ml produced a maximal increase in CO_2 production of 91% as compared to 133% for Triac. The stimulation by Tetrac, like that of Triac, occurs immediately and the rise is linear. Sarcoma 37 cells were also sensitive to Tetrac although the percentage stimulation was not quite as great as with the Ehrlich tumor.

Effect of Other Compounds on Aerobic Glycolysis—Two other substances, DNP and DIHPA, have been found to affect the Ehrlich carcinoma tumor cell and the Sarcoma 37 respectively. Of all the compounds tested, DNP produced the greatest stimulation of about 350% in twenty four minutes at a concentration of 1×10^{-3}M. DIHPA at 200 μg per ml induced a 46% stimulation of aerobic glycolysis.

TABLE 1. EFFECT OF TRIAC ON AEROBIC GLYCOLYSIS OF VARIOUS ASCITES TUMOUR CELLS

Type of cells and concentration in cells per flask	Time minutes	Triac μg per ml	μL CO_2	% Increase
Ehrlich Carcinoma 4.7×10^6	58	0	151	
		25	242	60
		50	353	133
		75	321	102
		100	270	78
Sarcoma 37 VS mouse 2.7×10^7	55	0	140	
		5	183	13
		10	203	45
		25	234	67
		50	294	110
		100	196	40
		200	130	−7
Sarcoma 37 C_3H mouse 2×10^7	124	0	400	
		25	450	13
		50	525	31
		75	400	0
		100	325	−19
		200	262	−34

* Correction of aerobic CO_2 production for oxygen consumption is not routinely presented in this note since it did not qualitatively alter the effects of the test compounds.

Triiodothyronine over a wide concentration range from 1 to 200 μg. per ml did not affect aerobic glycolysis of the Sarcoma 37 or the Ehrlich ascites tumor cells. Moreover, preincubation of the Ehrlich cells with the hormone for two hours at 22.5°C. did not result in an increase in rate over untreated cells. Under the same conditions of preincubation, Triac was still capable of accelerating the aerobic glycolysis. Differences in the effectiveness of thyroxine, or triiodothyronine, and the acetic acid analogues have also been observed in clinical studies on water balance and cholesterol metabolism (Lerman and Pitt-Rivers, 1955; Trotter, 1955).

Effect of Triac on Anaerobic Glycolysis and Oxygen Consumption—In a preliminary

TABLE 2. EFFECT OF TETRAC ON AEROBIC GLYCOLYSIS IN EHRLICH CARCINOMA AND SARCOMA 37 ASCITES TUMORS

Type of cell	Cells per flask	Time minutes	Tetrac μg per ml	μL CO_2	% Increase
Ehrlich Carcinoma	4.3×10^6	55	0	129	
			25	191	48
			50	246	91
			100	223	73
			200	171	33
Sarcoma 37	2.1×10^7	45	0	219	
			25	282	29
			50	306	40
			100	299	36
			200	231	5
Sarcoma 37	2.6×10^7	55	0	233	
			50	293	26

experiment we have found that 50 μg of Triac per ml produced about a 28% stimulation of anaerobic glycolysis in the Ehrlich cells. We have not as yet obtained stimulation anaerobically as large as that with aerobic glycolysis. Dr. S. B. Barker, who has confirmed our results on aerobic glycolysis in ascites tumor cells, found that a stimulation of anaerobic glycolysis also occurs with Tetrac (personal communication).

The effect of Triac on oxygen consumption is quite different from that on aerobic glycolysis. At low concentrations of Triac, between 1 and 15 μg. per ml., there is a small but reproducible stimulation, 10 to 15%, of oxygen uptake by the Ehrlich Carcinoma. At concentrations of Triac above 25 μg. per ml. there is an increasing inhibition of oxygen consumption. As in the case of aerobic glycolysis, the stimulation of oxygen uptake is an immediate effect. On the other hand, the inhibition is not linear and increases with time.

Triac and DNP appear to convert the tumor cell to anaerobic patterns of metabolism under aerobic conditions. In this respect the effect may be similar to the observations made by Lynen and Koenigsberger (1951) on the effect of DNP on fermentation and respiration of yeast.

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REFERENCES

THIBAULT, O. AND R. PITT-RIVERS: *Lancet* (i), 285. 1955.
NELSON, N.: *J. Biol. Chem., 153:* 375. 1944.
BARKER, S. B. AND SUMMERSON, W. H.: *J. Biol. Chem., 138:* 535. 1944.
LERMAN, J. AND R. PITT-RIVERS: *J. Clin. Endocrinol. and Metab. 15:* 653. 1955.
TROTTER, W. R.: *Lancet* (ii), 374. 1955.
LYNEN F. AND KOENIGSBERGER, R.: Liebigs Annalen der Chemie. *573:* 60. 1951.

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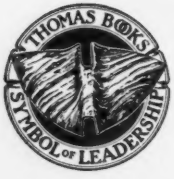

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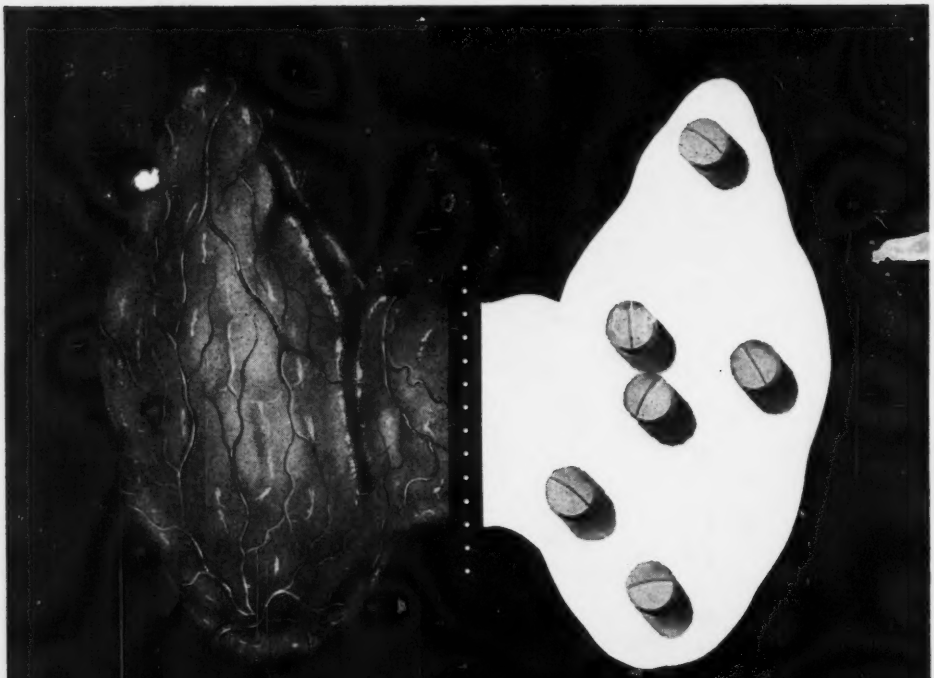

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